MASS REARING AND POPULATION GENETICS OF *ANOPHELES FREEBORNI*

By

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Techniques were developed for mass rearing Anopheles freeborni. Eggs were incubated, dried, and volumetrically dispensed into rearing trays. Water levels were kept low and at 28° C. Larvae were fed once daily on a slurry of liver powder, guinea pig chow, yeast and hog chow. Pupation started on the 7th day after egg hatch and were harvested on three consecutive days. Ca. 1,700 pupae were harvested/tray and 65% emerged as adults. Most sugar-fed males died after 2 weeks, whereas most females survived for 3 weeks. Colonies were maintained on defibrinated bovine blood provided in natural membrane prophylactics. Eggs were collected in plastic cups placed within cages; eggs could be dried and stored at 10 C for 6 days with only 6% mortality. A population genetics study of An. freeborni included a cytogenetic survey of polytene chromosome banding patterns and inversion frequencies, a determination of hybrid

incompatibility of different geographic strains, and a determination of genetic variability through analysis of 24 enzyme loci by starch electrophoresis. Population samples were obtained from California, Oregon and Washington. Four invariants were seen [In(3R)A, In(3R)C, In(3L)A, In(X)A] with frequencies that generally varied with geography or ecology. In(3L)A was most common in California and had similar frequencies throughout. Only populations collected in the Sacramento and Owens valleys, California, were fixed for the standard X homokaryotype. All other populations were either polymorphic for In(X)A or fixed for the inversion homokaryotype. A population at Jasper Ridge, California, was found to be An. hermsi (sibling species of An. freeborni) and had no invariants; both sibling species have homosequential chromosome banding patterns and the X chromosome does not distinguish them. Hybridization studies showed that only crosses with the Jasper Ridge strain produce hybrid sterility. The percentage of polymorphic loci ranged from 16.7-29.2 and mean heterozygosity ranged from 0.04-0.10. Two loci, Mpi-1 and Got-1, accounted for most polymorphism found in California; Pgm-1 was more polymorphic in Washington and Oregon, but Got-1 was fixed. Significant allele frequency differences between populations correlated with geographic distance or changes in ecology. No diagnostic loci were found that could distinguish An. hermsi from An. freeborni.

CHAPTER 1

INTRODUCTION

Throughout the past 20 years, a considerable amount of research has focussed on the genetics of mosquitoes. This focus has stemmed, in part, from the realization that many current methods of mosquito control are inadequate or no longer effective, and that genetics may offer a powerful tool for manipulating, and thus controlling, both mosquitoes and the diseases they vector. Furthermore, it has become apparent that understanding the genetic structure of insect vectors is fundamental to understanding the epidemiology of the diseases they transmit. The study of mosquito genetics, therefore, has proceeded on various fronts, each with its own approach.

Though considerable information on the cytogenetics of anophelines has accumulated during the past 2 decades (Kitzmiller 1976, Narang and Seawright 1989), literature on the population genetics and evolutionary relationships among Nearctic anophelines, particularly those in the maculipennis species complex, is scant and scattered. Studies on population genetic structure and phylogenetic relationships are useful in identifying vector versus non-vector populations and are also necessary first steps in certain types of control programs (e.g. population replacement). A

good example of the evolutionary and systematic problems now under investigation in culicids grew out of a very real and practical situation, the paradox of anopheliem without malaria in some parts of Europe. Although the supposed vector, An. maculipennis Meigen, was widely distributed, the distribution of malaria was not. Subsequent investigations showed that there were ecological differences among the various geographic races of An. maculipennis. Later, these races were found to correspond to seven species based on egg morphology, hybridization studies, and chromosomal comparisons. Thus, the maculipennis group became one of the classic examples of sibling species.

Pielou (1975) has emphasized the detection of cryptic species as of central importance in the study of ecology and natural history as well as in the genetic study of populations and their evolution. Species groups are common in the genus Anopheles and the Old World maculipennis group is simply one of many including the funestus, gambiae, punctulatus, leucosphyrus and minimus groups (see Reid 1970, Narang and Seawright 1989). Much less is known of the population genetic structure and phylogenetic relationships among the Nearctic maculipennis species complex. One difference between the Palearctic and Nearctic maculipennis species is that the latter show much greater chromosomal heterogeneity. This variation might indicate a New World origin of the maculipennis complex (Kitzmiller et al. 1967) and the presence of cryptic species. Recent investigations

by Lanzaro et al. (1988), Kaiser et al. (1988a, 1989b), and Narang et al. (1989a, 1989b, 1989c) have shown the presence of at least four sibling species for what was formerly thought to be one, An. quadrimaculatus Say.

The Nearctic maculipennis species group includes An. freeborni Aitken, An. aztecus Hoffmann, An. earlei Vargas, and An. occidentalis Dyar and Knab (Kitzmiller et al. 1967) Some authors consider An. quadrimaculatus and An. punctipennis (Say) as closely related, if not in this group too. An. freeborni has a widespread distribution in the states west of the Rocky Mountains extending from southern Canada to northern Mexico (Carpenter and LaCasse 1955). Recent studies indicate that all populations south of the Tehachapi Mountains and Santa Maria (San Luis Obispo Co.), California, may be those of a new sibling species, An. hermsi Barr (Barr 1988, Barr et al. 1987, Cope et al. 1988).

Anopheles freeborni is a serious pest in irrigated agriculture, particularly in California rice fields. Once an important vector of malaria in the U.S.A., An. freeborni is still a potential threat in sporadic malaria outbreaks (Singal et al. 1977). An. freeborni has also been found to harbor a number of arboviruses including western equine encephalitis, Cache Valley viruses, California encephalitis group viruses, ANA complex viruses and two strains of Main Drain virus (Reeves and Hammon 1944, Elbal et al. 1971, 1974, Calisher et al. 1980).

The little that is known about the genetics of An. freeborni concerns salivary chromosome mapping by Kitzmiller and Baker (1963), somatic chromosome size comparisons by Mukherjee et al. (1966), interspecific hybridization studies through forced copulation (see Kitzmiller et al. 1967), frequency of an inversion on chromosome arm 3K in three populations in California (Smithson 1970), and a comparison of alkaline phosphatases of An. freeborni and An. labranchiae Falleroni (Bianchi and Pirodi 1968). This work has provided some baseline information on the genetics of An. freeborni, but there is no information on the genetic structure and divergence of this species throughout its distribution range.

This investigation was funded by a grant from Walter Reed Army Institute of Research (WRAIR) to study the genetics of An. freeborni and to develop mass rearing techniques for this species. WRAIR and other institutions currently involved in malaria vaccine development are particularly interested in mass rearing An. freeborni because this species has consistently high rates of infection with malaria sporozoites and oocysts under laboratory conditions; it is the ideal species for malaria transmission research and studies involving the use of oocysts and sporozoites. Furthermore, relative to other Nearctic anophelines, An. freeborni has been found to have higher rates of infection with both Plasmodium falciparum and P. vivax (including various strains of these), and a

number of zoonotic malarias (Collins et al. 1979, 1980, 1981, 1982, 1983, 1984). At present, there is no method for the in vitro culture of the mosquito stages of malaria.

The purpose of this investigation, therefore, has been twofold: first, to develop a methodology that is suitable for rearing large numbers of An. freeborni, and second, to study the population genetic structure and genetic divergence of An. freeborni over widely separated geographic areas. It is clear from past studies of mosquitoes, particularly the anophelines of S.E. Asia (Reid 1968) and Africa (Coluzzi et al. 1979), that most wide-ranging species vary geographically. This variation may take the form of morphological differences (often enough to require region-specific keys), ecological differences, and vector capacity differences (Reid 1970). Although Depner and Harwood (1966) reported differences in photoperiod response between two latitudinally-distinct groups of An. freeborni, no other studies have investigated differences between spatially distant strains of this species.

Three complementary approaches have been used in this investigation to study the population genetics of An. freeborni: enzyme electrophoresis, cytogenetics, and hybridization between different populations. It was hoped that these three methods would be useful in answering and elucidating the following:

1. a measure of genetic polymorphism (polymorphic enzyme loci, heterozygousity, inversion

frequencies and distributions) in natural populations;

2. genetic distances between conspecific populations;
3. diagnostic enzyme loci for species and populations;
4. the presence of a sibling species complex;
5. insights into speciation, genetic-environmental correlations and phylogenetic relationships.

This thesis is divided into six chapters. The second chapter describes the techniques developed for mass rearing An. freeborni. The last four chapters are devoted to cytogenetics, hybridization, enzyme electrophoresis, and a general discussion.

CHAPTER 2

IMPROVED TECHNIQUES FOR REARING ANOPHELES FREEBORNI

Introduction

Anopheles freeborni Aitken is a member of the North American maculipennis group and is found west of the Rocky Mountains (Carpenter and La Casse 1955). It is a major pest in certain areas, primarily in irrigated lands, and was once the most important vector of malaria on the West Coast. Because of its relatively high rates of infection with various strains of human malaria (Burgess and Young 1950, Collins et al. 1964, 1973a, 1973b, 1977,1981), An. freeborni has been the preferred domestic species in malaria research.

Techniques for rearing An. freeborni have been published by Hardman (1947), Depner and Harwood (1966), Miura (1970) and Garberg (1970); laboratory observations on developmental biology were described by Northup and Washino (1981). Their techniques, and other methodologies presently used at several research institutes in the US, are laborious, imprecise and concerned with producing relatively small numbers of mosquitoes for laboratory tests. The current study, made possible by funding from the US Army Medical Research and Development Command, was conducted to develop techniques for mass production where time, labor and expense

can be limiting factors. An extensive amount of research has been conducted at the USDA, Insects Affecting Man and Animals Research Laboratory (IAMARL), Gainesville, FL, on the mass rearing technology of An. albimanus Wiedemann and An. quadrimaculatus Say (Dame et al. 1974, 1978, Bailey et al. 1978, 1979a, 1979b, 1980a, 1980b, 1980c, Fowler et al 1980, Savage et al. 1980). The technology developed has made possible the continuous provision of large numbers of mosquitoes for various research projects at the IAMARL. The purpose of this project was to develop mass rearing methodology for An. freeborni.

Materials and Methods

Rearing Facility

A rearing room was established in the quarantine unit of the Florida Department of Plant Industry in Gainesville. The room was equipped with a humidifier and heating system to provide a relative humidity of 70-80% and a temperature of 25-27°C. A 12:12 light:dark (LD) cycle was maintained, and a timed 4-watt night light provided a 2-hour crepuscular period. Four metal racks, each capable of holding 20 plastic rearing trays (51 X 38 X 8 cm), were housed in 2 cabinets that had clear plastic sliding doors. The cabinets helped maintain a constant water temperature in the larval holding trays, reduced evaporation of water from trays and inhibited access to the trays by loose mosquitoes. The water temperature in the trays was controlled with

electrical heat tapes (Dame et al. 197d) that ran the length of each shelf of each rack and were controlled by Zipcon® variable temperature controllers.

Adults

Adults were held in 61 X 61 X 61-cm aluminum-frame screened cages with tubular sleeving attached to one side. The bottom and top of each cage was covered with white Foreica® to facilitate cleaning. Two feeding ports located on the top panel (Bailey et al. 197d) made it possible to provide sugar and blood without placing hands or arms in the cage. Cotton soaked in a 10% sugar solution was provided twice a week.

Mass rearing techniques were developed using a strain of An. freeborni obtained from Dr. Robert Washino, University of California, Davis. This strain (Davis strain) originated from mosquitoes collected in the Sacramento Valley, and had already been maintained for ca. 2 years as a laboratory colony prior to this investigation. The mass rearing methodology developed for the Davis strain was tested on a 2nd strain of An. freeborni that was established from the eggs of 52 field-collected females from Benton County, Washington. The Washington strain was in its 6th generation at the time of the mass rearing tests.

To facilitate colony establishment and maintenance, the mating behavior of virgin males and females was investigated by combining 50 pairs of the following individuals in

gallon-sized containers with screen tops: (1) teneral females and males; (2) teneral females and 5-day-old males; (3) 5-day-old females and 5-day-old males; (4) 5-day-old females and teneral males. There were 18 replicates. Every day, 5 females were removed from each container and their spermathecae examined for the presence of spermatozoa.

To test whether individual males would inseminate multiple females and copulate in the absence of swarming, single virgin teneral males were placed in gallon containers with 1, 5 and 10 virgin teneral females. female spermathecae were dissected when males died.

Bloodfeeding

Initially, all mosquito strains were fed on restrained guinea pigs or on human arms. Once each strain was established in large numbers, bovine blood was tested as an alternative bloodmeal. Bovine blood was obtained weekly or bi-weekly from a local slaughterhouse, immediately defibrinated mechanically, and refrigerated at 2-5°C. Mosquitoes were fed through pre-processed natural membrane prophylactics in the manner described by Bailey et al. (1978).

To test the acceptability of the membrane prophylactics in terms of feeding preference, 3 groups of 25 six-day-old female mosquitoes were fed for 15 min on the shaved bellies of restrained guinea pigs, and 3 other groups were fed on membranes containing 100 ml of defibrinated bovine blood.

Guinea pigs, cages and membranes were completely randomized in 9 replicates conducted on 9 separate days; on 2 of these days only 4 cages were used, and on 1 day 5 cages were used.

Since the type of bloodmeal ingested by female mosquitoes can effect egg production, clutch size was compared for females fed on guinea pigs versus those fed on defibrinated bovine blood. One hundred female and male pupae were chosen randomly from a single day's harvest and isolated by sex. Six days after emergence, two groups of 35 females were allowed to feed on either a guinea pig or on defibrinated bovine blood (24 hr old and heated to 40 C) for 15 minutes. Bloodfed females, and an equal number of males, were subsequently transferred to gallon-sized containers and held for 3 days. Females were then transferred to individual vials lined with filter paper and containing water. The number of eggs and percent hatch was recorded for each female. A 2nd replicate of this study also included a comparison with whole human blood.

Egg Drying and Storage

Eggs were collected by placing plastic cups filled with water into the cages. Eggs deposited the previous night were first washed through a screen into an enameled pan to remove any dead adults. The enameled pan containing the eggs was then placed into the rearing cabinet and incubated for ca. 24 hr at a water temperature of 28°C; heat tapes maintained a constant water temperature. After incubation,

eggs were dried and volumetrically measured in the manner described by Dame et al. (1978). By dispensing measured quantities of dried eggs into the rearing trays, it was possible to standardize the number of larvae per tray. Since drying might affect egg hatch, the percentage hatch of 10 dried samples of 800 eggs each was compared to that of 10 non-dried samples. All eggs originated from the same day's batch.

The ability to dry and store eggs makes it unnecessary to collect eggs daily from rearing cages, allows for their easy shipment, and provides for an emergency stockpile. The effect of storage on egg hatch was tested at -20, 5, 10, 15 and 26°C. Eggs from the same day's batch were divided into samples of ca. 500 and placed in individual plastic Eppendorf microcentrifuge tubes (1.5 ml). Thirty such samples were stored at each temperature. There were 10 replicates at 5, 10 and 15°C and 4 replicates at -20 and 26°C. Three samples of eggs from every batch were allowed to hatch immediately as controls. Thereafter, 3 samples of eggs were removed from each treatment at 3-day intervals. All eggs were hatched in styrofoam cups containing 50 ml of an infusion made by mixing 0.02 g of a 1:1:1 mixture of liver powder, yeast and hog supplement in 75 ml water; the infusion was strained through organdy cloth to remove large particles. Percentage hatch was determined by examining 300 eggs from each sample at 10X magnification; hatched eggs were distinguished by their collapsed chorions and opened

operculums. Since the percentage hatch for controls varied between replicates, Abbott's (1925) correction formula was employed prior to the analysis of variance.

Various concentrations of glycerol and dimethylsulfoxide (DMSO) were tested for their affects on the storage of dried eggs and 1st instar larvae at -20°C. Mosquitoes were also allowed to deposit their eggs on water containing different concentrations of both substances. All larvae and eggs were stored in 1.5 ml microcentrifuge tubes.

Larval Rearing

Various rearing conditions were tested that included changes in the type and quantity of food, volume of water, and density of larvae. The types of foods tested were Agway and Gaines dog food (de-fatted), hog supplement, guinea pig and fish chow (Ralston Purina Co.), desiccated hog liver powder, and brewer's yeast (ICN Pharmaceuticals Inc). The dog food, hog supplement, guinea pig and fish chow were sieved through a No. 50 sieve. Food was provided to each rearing tray in the form of a surface dust or as a slurry which was mixed into the rearing water. The following criteria were used individually or collectively to determine the effectiveness of the various rearing techniques: time for development, number and weight of pupae, percentage adult emergence, adult longevity and sex ratio.

All larvae were reared in plastic trays (51 X 38 X 8 cm) with tap water that was not dechlorinated. Larval densities that were tested ranged from 2,000 to 5,000

individuals per tray. Pupae were harvested by utilizing the cold water technique of Weathersby (1961) as modified by Hazard (1967). All pupae were counted, and samples of 100 individuals from each tray were sexed and weighed on days of maximum pupation. Prior to weighing, pupae were surface-dried by blotting with tissue paper. Daily harvests of pupae were put into plastic cups with clean water and placed in emergence cages. Plastic funnels over the cups prevented females from entering the cups and laying their eggs, but allowed emerging adults to escape.

Adult emergence and longevity, under the rearing methodology established in this study, were estimated from samples of 100 pupae that were selected randomly from each of 32 rearing trays. Each sample was weighed, placed in a styrofoam cup with 50 ml water, and allowed to emerge in gallon-sized containers with screen lids. Dead mosquitoes were counted, sexed, and removed from each container every day.

The length of time for development of pupae into adults was determined by isolating 50 newly pupated individuals of each sex in gallon-size containers. Each day thereafter, the number and sex of emerged adults was recorded. There were six replicates and all pupae in each replicate were drawn from a single day's batch of newly-pupated individuals.

Results

Both strains of An. freeborni fed readily on defibrinated bovine blood contained in natural membrane prophylactics. Defibrinated blood could be stored at 0-5°C for 10 days before mosquitoes refrained from feeding on it. The mean number of females that blood-fed when offered either a guinea pig or defibrinated bovine blood was identical, out of 51 cages of mosquitoes tested, a mean of 15.0 ± 4.5 and 15.5 ± 3.1 females per cage (25 females/cage) took bloodmeals from guinea pigs and membranes, respectively. Furthermore, females that fed on defibrinated blood produced as many eggs with a similar percent hatch as those fed on guinea pigs or human blood (Table 2-1).

Table 2-1. Mean number (± SD) of eggs and percentage hatch for females fed on guinea pigs (GP), defibrinated bovine blood (DEF) and a human arm (HUM).

Rep.	Blood	n	No. eggs/female	% Hatch
I	GP	20	128[ab] ± 29.6a	69.7 ± 22.0a
	DEF	17	104 ± 36.0a	67.5 ± 29.7a
II	GP	36	155 ± 53.1b	96.0 ± 7.4b
	DEF	23	149 ± 50.6b	95.3 ± 3.7b
	HUM	24	138 ± 46.7b	97.8 ± 2.6b

[ab] Means in the same column and replicate followed by the same lower case letter are not significantly different (P = 0.05).

Among teneral mosquitoes of both sexes, insemination did not occur until the 3rd day post-emergence (Fig. 2-1). When teneral individuals were caged with 5-day-old mosquitoes of the opposite sex, spermatozoa were found in 20-40% of the females within 24 hr. On the other hand, when 5-day-old mosquitoes of both sexes were caged together, almost 90% of the females were inseminated within 24 hr. These observations suggest that the absence of any mating among teneral mosquitoes during the 1st two days post-emergence is due, in part, to the behavior of both males and females. Additional mating tests demonstrated that teneral females that were bloodfed on the third day post-emergence (females will not feed until at least three days old) had the same rate of insemination as tenerals that were not bloodfed; in effect, a bloodmeal was not a necessary condition for insemination nor did it effect the rate of insemination.

The genitalia of newly emerged males (n = 35) were observed to rotate fully in ca. 20 hr at 25°C. Genitalia rotated 180° in a clockwise or counterclockwise direction with apparently equal frequency: 26 out of 58 individuals rotated clockwise.

Attempts to rear many other species of mosquitoes have often failed or been very difficult due to the absence of mating among adults in laboratory cages. For some species, the appropriate conditions that trigger swarming by males appears to be critical for ensuring copulation.

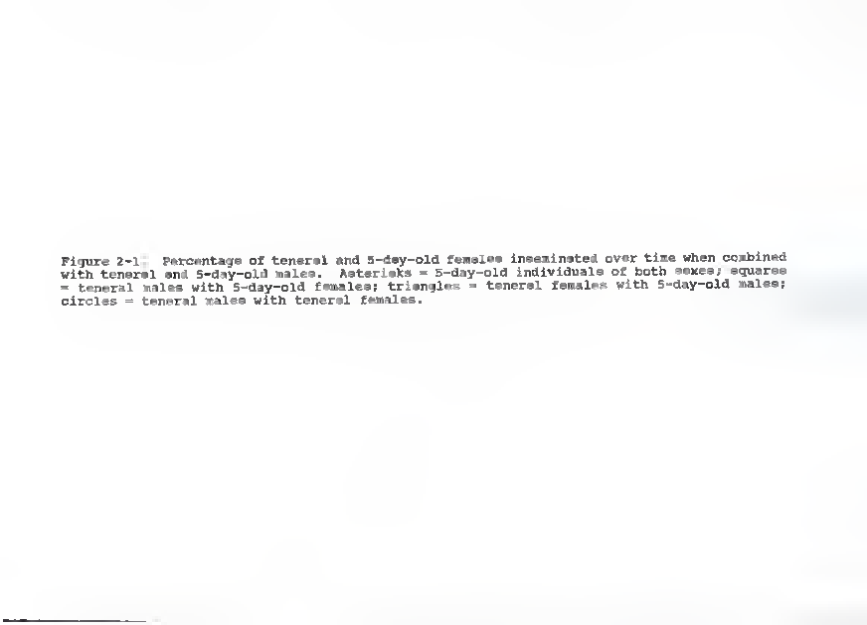
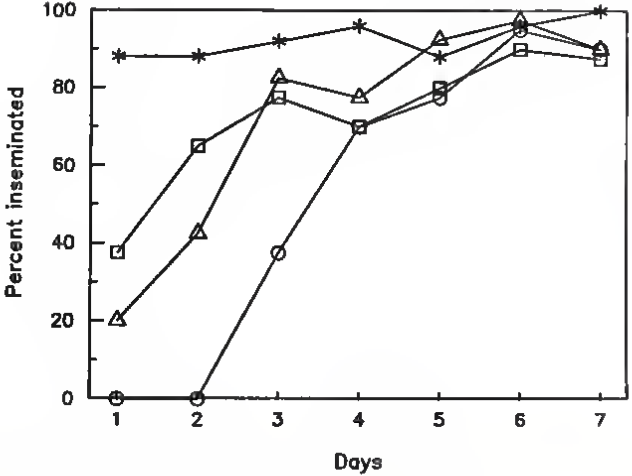


Figure 2-1. Percentage of teneral and 5-day-old females inseminated over time when combined with teneral and 5-day-old males. Asterisks = 5-day-old individuals of both sexes; squares = teneral males with 5-day-old females; triangles = teneral females with 5-day-old males; circles = teneral males with teneral females.


Percent inseminated
100
80
60
40
20
0
1
2
3
4
5
6
7
Days

Fortunately, this is not the case with An. freeborni. Single males held in gallon-size containers with varying numbers of females did copulate (Table 2-2). One male copulated with 10 females.

Females usually laid their eggs on the 3rd or 4th day after blood-feeding. The eggs were deposited on water contained in plastic cups of various sizes, irrespective of their color (white or painted black). After a 24-hr incubation period, eggs were dried and dispensed into trays using the techniques described by Dame et al. (1978); it was not necessary, however, to hatch the eggs in small cups with

Table 2-2. Inseminations by single males placed with 1, 5 or 10 virgin females in individual gallon-sized containers

No. females	Reps	Inseminations: Number Total	Inseminations: Number Range	Inseminations: %
1	10	5	0-1	50
5	10	23	0-4	46
10	10	49	1-10	49

infusion water before adding the larvae to large trays. Rather, dry eggs were sprinkled into 5-cm-diam styrofoam rings floating in each tray. Crowding of the eggs in the

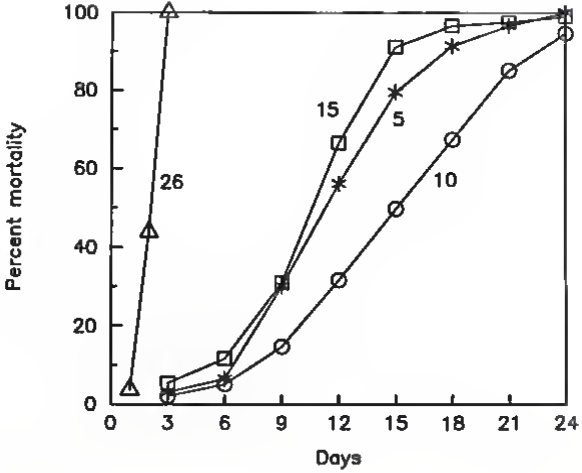
floating rings was done to encourage hatch synchrony (Dame et al. 1978).

A volume of 0.01 ml was estimated to contain ca. 783 ± 56 dry eggs. Egg hatch for dried eggs (83 ± 3%) versus non-dried eggs (85 ± 4%) did not differ significantly (P = 0.05), nor was a reduction in hatch or increase in larval mortality apparent after eggs were dried routinely.

Attempts to store eggs at -20°C for any length of time, whether dry or in various concentrations of glycerol and DMSO, were not successful (complete mortality). Dry eggs could be stored, however, at temperatures above 0°C (Fig. 2-2). Mortality of dried eggs stored for 6 days at 5, 10 and 15°C ranged from 2.1 to 11.7%. Mortality did not differ significantly at these temperatures during the 1st six days of storage, but thereafter mortality for eggs stored at 10°C was consistently and significantly lower (P = 0.05). Even after 18 days of storage at 10°C, the percentage mortality was only 67.4. Eggs stored at room temperature for 24 hours (26°C) survived remarkably well, but none hatched when stored at this temperature for more than 2 days.

When An. freeborni larvae were reared with amounts of water/tray and types of diets identical to those used for mass rearing An. albimanus (see Dame et al. 1978) and An. quadrimaculatus at IAMARL, the water clouded and all larvae died. Observations on the feeding behavior of the latter two species in colony at IAMARL revealed that all larval instars fed on the bottom of trays as well as on the

Figure 2-2 Percent mortality of dry eggs stored at 5, 10, 15 and 25 C. All mortality values were transformed using Abbot's formula


Percent mortality
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
Days
26
15
5
10

surface. Anopheline mosquitoes are generally thought to be surface feeders, and perhaps the colonies at IAMAR have been selected over the years for bottom feeding. An. freeborni in this study were seen to swim and feed at the bottom of rearing trays only during the 1st larval instar In subsequent instars, larvae remained on the water surface to feed. Consequently, the depth of water/tray was reduced with the result that larvae were then able to feed on food settled on the bottom of trays. Larvae were observed to angle their bodies downward and graze, while still maintaining their attachment to the water surface. Of the various water levels tested, an initial amount of 500 ml/tray, supplemented with additional water at each daily feeding (Table 2-3), allowed larvae to graze on tray bottoms throughout most of their development. With this approach to feeding the larvae, it is particularly important that tray bottoms be uniformly flat and that the shelves on which they sit be adjusted with a level. Otherwise, food collects in the deeper portions of the trays, becomes inaccessible to larvae, and eventually fouls the water.

The diet that caused the least amount of fouling of water was a mixture of 3 parts guinea pig chow to 1 part each of liver powder, yeast and hog chow. When trays are set on the 1st day with dried eggs, 0.1 g of food is dusted on the water surface (Table 2-3). Thereafter, the food is combined with a given amount of water in a slurry and mixed into the rearing water

Table 2-3. Daily diet and stage of development under standardized rearing procedure.

Day	Diet (g)	Stage
1	0.1 dust	eggs
2	0	hatch
3	0.5 in 50 ml	larvae
4	0.5 in 50 ml	"
5	1.5 in 100 ml	"
6	1.5 in 100 ml	"
7	2.0 in 100 ml	"
8	2.5 in 100 ml	"
9	1.0 in 100 ml	pupation
10	0.5 mixed in	1st pick
11	0.5 mixed in	2nd pick
12	0	3rd pick

All trays were started with 3,917 ± eggs in 500 ml water. On the third to ninth days, all food was mixed with water and added as a slurry to each rearing tray. On the 10th and 11th days, 0.5 g was added to the surface as a dust and then mixed into the water by shaking the tray back and forth a few times. With 0.05 ml of dried eggs (3,917 ± 280), 500 ml of initial water/tray and a total of 10.6 g of food, an average of ca. 1,700 pupae/tray was produced from 49 trays

Table 2-4. The mean number, weight (wgt. = mg/100) and sex ratio (F = % females) of pupae harvested on 3 consecutive days under standardized rearing procedures. These are based on 2 replicates with a strain from California and 1 with a strain from Washington. All trays were started with 3,917 + 280 eggs in 500 ml water.

			Pupae							
			1st			2nd			3rd	
Strain	No. Trays	% Hatch	No.	Wgt.	%F	No.	Wgt.	%F	No.	Total
Cal I	19	87a*	562b	341e	30a	831e	388b	54b	379e	1772a
II	18	83b	794a	328b	33e	695b	305b	81a	302e	1790a
Wash	12	82b	455b	368c	29a	795ab	341e	56ab	399e	1649e

*Means in the same column followed by a different lower case letter are significantly different (P = 0.05).

Table 2-4). Stocking the trays with fewer or more eggs but with the same proportion of food per larva generally led to larger and less numerous pupae or smaller and more numerous pupae, respectively.

Pupae first appeared on the 7th day after egg hatch and were harvested on the following 3 days (Table 2-3). Thereafter, the number of individuals pupating dropped and was not very synchronous. Males develop faster than females and account for the skewed sex ratio of pupae on each day they were harvested (Table 2-4). There did not, however, appear to be any difference in the duration of development between male and female pupae (Table 2-5). Males probably pupate first because of a faster embryonic or larval development. There were no significant differences in the total number of pupae harvested for each replicate of larval rearing or between 2 strains of An. freeborni. The mean weight per 100 pupae was well over 300 mg, and over 40% of all individuals harvested were female.

Adult mosquitoes emerged from 85% of the pupae (n = 3,200). Thirty-five percent of the adult females, fed only on sugar water, survived for three weeks. Male mosquitoes, however, were much shorter lived-- only 10% survived for 2 weeks (Fig. 2-3).

Table 2-5. Mean percentage (± SD) emergence of adults per day; all pupae were between 0-24 h old and divided into six groups of 50 individuals/sex.

			% Emergance/day		
Sex	n	Total % emerg-	1	2	3
M	300	94.3 ± 3.2	0.7 ± 1.1	67.2 ± 6.9	100
F	300	94.0 ± 4.6	0.4 ± 0.9	59.1 ± 6.5	100

Discussion

Hundreds of tests were conducted, in which larval densities and nutrition were varied, before an acceptable diet was established. Dusting fine particles of food on the water surface of larval rearing trays has been a standard method for maintaining anophelines including An. freeborni This technique, however, proved to be unsatisfactory for mass rearing technology for several reasons. First, the amount of food that can be added to a tray is limited by the surface area of the water and the layer of food that larvae can tolerate. If too much food is added, the larvae cannot penetrate the layer and suffocate. Furthermore, bacteria and yeast can quickly form a scum on the surface which can also kill larvae. When larval densities in a tray are low,

Figure 2-3. Percent survival of adult female and male Anopheles freeborni fed on sugar water (male = crosshatch, female = solid).

Percent survival
100
80
60
40
20
0
1
2
3
Weeks

the amount of food allowable on the surface is usually enough to avoid starvation. At higher larval densities, however, the same surface area cannot provide enough food for the increased demand. Furthermore, the demand for food increases as the larvae develop into later instars. Consequently, high densities of larvae can be maintained on a surface dusting regime only by adding small quantities of food several times a day. Invariably, much of the food settles to the bottom of the tray and fouls the water. Typically, as soon as the rearing water shows signs of becoming cloudy, the larvae die or are delayed in their development.

After 3 harvests of pupae per tray in this study, the rate of recovery from 1st instar larvae was between 51-55%. This rate is comparable to the total recovery rates obtained by Hardman (1947) (58%) and Northrup and Washino (1981) (52%). Consolidation of the remaining larvae after the 3rd harvest of pupae (see Fowler et al. 1980) could further increase the yields of pupae per tray. Furthermore, the rearing techniques developed here make it possible to rear larvae at 7 times the densities reported by Hardman (1947) and with almost half the development time. Although larval densities could be further increased by adding more eggs to each tray, overcrowding appeared to reduce not only the percentage of larvae pupating, but also adult emergence and pupal weight; similar effects have been reported by Krishnan et al. (1959) for Culex fatigans Wiedemann and by Terzian

end Stahler (1945) for laboratory-reared An. quadrimaculatus.

For malaria research involving the production of oocysts and sporozoites, it is important that female mosquitoes survive between 2-3 weeks. With the rearing methodology described for An. freeborni in this study, between 40-70% of the females can be expected to survive for this period of time.

At least some strains of An. freeborni are stenogamous (i.e., will copulate in small containers) under laboratory rearing conditions, and it can be easy to start a colony from a few field-collected individuals. Since individual males will often mate with one or more females, this species is particularly suitable for genetic studies.

During this investigation, An. freeborni was collected from Utah, Washington and several locations in California. For the most part, few problems were encountered in establishing each strain as a laboratory colony. Two strains have been maintained for 60 generations. There were, however, some problems associated with particular strains that are worth mentioning. Initially, attempts to develop a rearing methodology for An. freeborni were made by using the Marysville strain (obtained from Walter Reed Army Institute of Research) that had been in colony for 45 years. Although larvae and pupae appeared to develop normally, adults did not emerge properly and died. Only later did it become apparent that this strain was temperature sensitive

and could not be reared at a water temperature of 28°C or more. This strain could be reared successfully at 25-26°C (although under a different feeding regime than that outlined above). Another strain that was difficult to rear originated from Utah. Females collected in Utah during the fall would take a bloodmeal, but would not lay eggs. These females may have already undergone gonotrophic dissociation, a type of facultative diapause characterized by the suspension of reproductive activity (Washino 1970). Two strains collected in April in the Sacramento Valley, California exhibited very low rates of inseminated females during the first few generations.

In conclusion, the capacity to produce large numbers of mosquitoes efficiently is a pre-requisite for many malaria research and mosquito control programs. With the methodology described above, it is now possible to mass-rear An. freeborni. The number of larvae per tray is standardized by volumetrically measuring eggs, water temperature is kept constant with heat tapes, rearing trays do not need to be subdivided as larvae mature, high densities of larvae can be maintained, and larvae are fed only once a day. Adults can be maintained on defibrinated bovine blood, thus eliminating the need to keep live animals.

CHAPTER 3

CYTOGENETIC SURVEY OF ANOPHELES FREEBORNI

Introduction

Many anophelina mosquitoes possess ovarian nurse cell and salivary gland polytene chromosomes that are generally very good for cytological observations. The banding pattern of the polytene chromosomes is a direct expression of gene arrangement, and can provide reliable indications of intra- and interspecific differentiations and phylogenetic relationships (Dobzhansky and Sturtevant 1938). Banding patterns and inversions in polytene chromosomes have been extremely important in describing and distinguishing sibling species, and in tracing evolutionary relationships in both the Anopheles gambiae and maculipennis groups (Coluzzi and Sabatini 1967, 1968, Frizzi 1947, 1951, 1952). Cytotaxonomic identification has been used to investigate the ecological, seasonal and geographic distributions of members of the gambiae group (Coluzzi et al. 1979, 1985) and was used to distinguish vector versus non-vector populations of An. nuneztovari Gabaldon (see review by Kitzmiller 1976).

This is the first comprehensive study of the polytene chromosomes of wild populations of An. freeborni over a broad range of this species' distribution. The purpose of this study was twofold: 1) to record the presence and

frequency of inversions; 2) to detect the possible presence of cryptic species by comparing banding patterns and inversions within and between populations. While this investigation was in progress, An. hermsi Barr was described (Barr 1988) as a sibling species of An. freeborni. According to the description, the only reliable way of distinguishing these two species is by their polytene X chromosomes; they differ by one simple inversion (Fig. 3-1). Consequently, it became particularly important in this study to identify the X chromosomes of individuals from all populations presumed to be An. freeborni. The results of this cytogenetic survey of An. freeborni invalidated the use of the X chromosome as a character to distinguish the two sibling species. Therefore, the following introduction is included to serve as a short review of the history and taxonomic status of An. hermsi.

Cytogenetics of Anopheles freeborni

Frizzi and DeCarli (1954) were the first to describe certain areas of the salivary gland chromosomes of An. freeborni that were homologous in banding pattern to those of An. atroparvus Van Thiel. Kitzmiller and Baker (1963) then published a complete description and map of the salivary polytene chromosomes of An. freeborni. Foran (1981) described the banding pattern of the ovarian nurse cell polytene chromosomes and also prepared a corresponding map. All of the above investigators used the same strain of

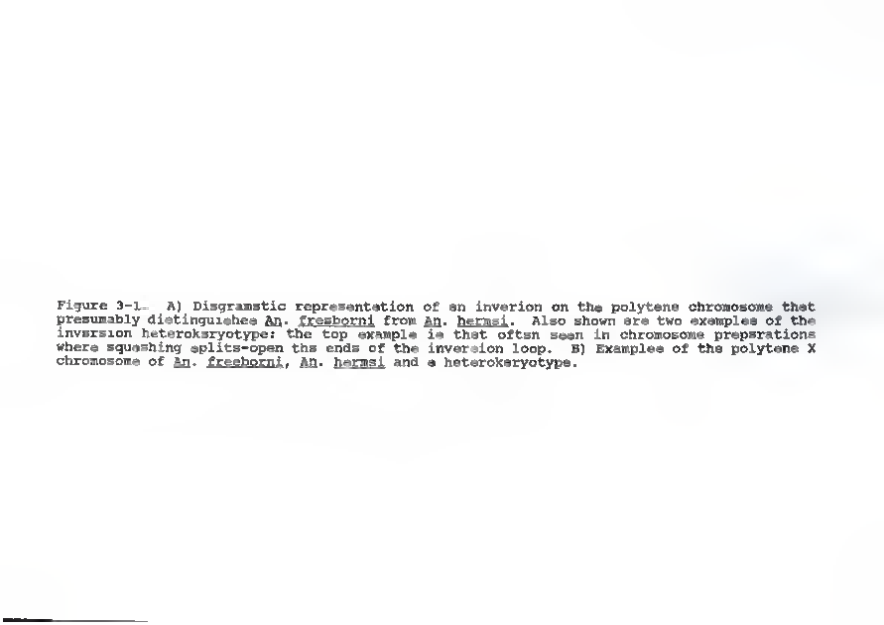


Figure 3-1. A) Diagramatic representation of an inverion on the polytene chromosome that presumably distinguishes An. freeborni from An. hermsi. Also shown are two examples of the inversion heterokaryotype: the top example is that often seen in chromosome preparations where squashing splits-open the ends of the inversion loop. B) Examples of the polytene X chromosome of An. freeborni, An. hermsi and a heterokaryotype.

A
B
An. hermsi
An. freeborni
Heterokaryotype

An. freeborni, which originated in Marysville, California (Herdman 1947), and is currently maintained in several laboratories in the United States including WRAIR. Baker (1965) described the mitotic chromosomes and Mukherjee et al. (1966) made comparisons of somatic (brain tissue from larvae) chromosome size.

Chromosomal aberrations

Frizzi and DeCarli (1954) described only two inversions in An. freeborni--one on each arm of chromosome 3. In(3R)A occurred at subzones 29B-31A, whereas In(3L)A continued from 36A to the centromere (covers ca. 2/3 length of the arm). Both inversions were relatively common in their laboratory colony. Kitzmiller and Baker (1963) studied ca. 300 slides of salivary chromosomes prepared from a laboratory strain and found no inversions or duplications in the X chromosome. Chromosome 2 had no inversions, but had a rare duplication in subzones 18A-18B. Chromosome 3 had a frequent inversion that occurred on the right arm at subzones 29B-31A. An inversion on 3L (subzone 36A-centromere) was only seen once and no homozygote was ever recovered. Feron (1981), who studied the ovarian nurse cell chromosomes, reported finding an inversion on 3R only. This inversion was between subzone 29B-30E and is almost certainly the same inversion that Kitzmiller and Baker (1963) and Frizzi and DeCarli (1954) described for 3R in the salivary chromosomes; Feron (1981) used a zone numbering system homologous to that used by

Kitzmiller and Baker (1963) and Frizzi and DeCarli (1954). Menchaca (1986) reported three inversions on chromosome 3 from a sample of 50 salivary gland preparations; her laboratory strain originated from a collection made in the Davis, California area. All three inversions apparently start at subzone 29A and end proximally to 30E. According to her description, In(3R)A was identical to that described by Kitzmiller and Baker (1963), whereas In(3R)B and In(3R)C were previously unknown. In(3R)C is formed by two included inversions. Menchaca (1986), however, never saw the inversion homokaryotype for In(3R)B and did not mention how this inversion differs from In(3R)A; its existance, therefore, is questionable, particularly since the inversion begins and ends in the same subzones as In(3R)A! Menchaca (1986) found no other inversions in the chromosomes of An. freeborni. All studies to date, therefore, have found that An. freeborni has polymorphic inversions on chromosome 3 only: one inversion on 3L, and at least two inversions on 3R.

There has been only one cytogenetic study of the polytene chromosomes of wild populations of An. freeborni Smithson (1970) attempted to compare the inversion polymorphism in different populations and determine whether there were seasonal intrapopulation fluctuations in inversion frequency. He sampled three populations within the Sacramento Valley and made over 800 preparations of the salivary gland chromosomes. The only inversion he reported

was on 3R between subzones 29A- 31A (In(3R)A). Unfortunately, Smithson's (1970) preparations were so poor that he had to abort the project and was only able to record the frequency of the heterokaryotype. Only 6% of his chromosome preparations were readable, and he was not able to distinguish either homokaryotype.

Cytogenetics of Anopheles hermsi

Anopheles hermsi is a sibling species of An. freeborni and was described recently by Barr (1988). According to Barr et al. (1987) and Cope et al. (1988), An. hermsi is known only from certain areas in northern Mexico and parts of California south of Santa Maria (San Luis Obispo Co.) and the Tehachapi Mountains.

Over the past 45 years, the taxonomic status of this species has been in contention. Early records of "An. freeborni" in southern California showed its distribution limited to the coastal regions from Santa Barbara to Baja California (Aitken 1939, 1945). Along the coast North of Santa Barbara, An. freeborni appeared to be displaced by An. occidentalis Dyar and Knab. Since the southern coastal populations of "An. freeborni" appeared to be identical morphologically to An. freeborni from the Sacramento Valley, Aitken (1945) considered them to be conspecific. Aitken (1939, 1945) did acknowledge the possibility that the populations south of Santa Barbara were merely southern forms of An. occidentalis that had lost their pale wing-tip

markings (the character that distinguishes An. freeborni from An. occidentalis). Freeborn and Bohart (1951), in their bulletin on the mosquitoes of California, chose to combine (for no reason stated) the southern coastal populations into the species An. occidentalis.

Lewallen (1957) was the first to refer to the southern coastal populations as "southern occidentalis". Lewallen (1957) compared An. freeborni from the Sacramento Valley to both "southern occidentalis" and An. occidentalis by using ninhydrin-positive paper chromatography. In my opinion, his study was very preliminary, the sample size was too small, and the results were confusing. The numbers, types, and Rf values (relative mobilities) of the ninhydrin spots varied between and within each "species" and between each sex. Since "southern occidentalis" had a total number of spots closer to that of An. occidentalis, Lewellen (1957) suggested, incorrectly, that these were conspecific and the southern form had simply lost its pale wing spots. Rather than show a close relationship between An. occidentalis and "southern occidentalis", Lewallen's (1957) data is actually best interpreted as showing close affinity of the latter with An. freeborni. Every ninhydrin spot present in "southern occidentalis" is also present in An. freeborni, whereas "southern occidentalis" only shares from 30-75% of its spots with An. occidentalis.

Kitzmiller and Baker (1963) made the next reference to "southern occidentalis" in their study of the X chromosomes

of the salivary glands of certain anophelines. They concluded that "southern occidentalis" and An. freeborni had an unmistaken homology of their X chromosomes as evident in the pairing of hybrid heterozygotes. At this time, Baker and Kitzmiller (1963) had not yet studied the X chromosome of An. occidentalis and, therefore, could not make any conclusions regarding the southern and northern forms of this supposed single species. Later, Baker (1965) studied the salivary gland chromosomes of all the Nearctic maculipennis complex and clearly distinguished "southern occidentalis" from An. occidentalis on the basis of both the X chromosome and the autosomes. Baker (1965) also hybridized An. freeborni with "southern occidentalis" and found that there was complete pairing between the autosomes except possibly near the centromere regions. The only difference between the two "species" was a simple fixed inversion on the X chromosome (InX) at subzones 2B-5C (Fig 3-1). Baker (1965) then suggested that the name "southern occidentalis" be dropped, since its affinity was clearly to An. freeborni.

Morrison (1985) described and produced a map of the ovarian nurse cell polytene chromosomes of "southern occidentalis". Menchaca (1986) did the analysis of the salivary gland chromosomes. Menchaca (1986) also repeated Baker's (1965) crosses to An. freeborni and described the hybrid polytene chromosomes. Fujioka (1986) studied the fecundity, fertility, progeny viability and sterility of

crosses between An. freeborni and "southern occidentalis". When the male parent was An. freeborni, all F1 males were sterile. In the reciprocal cross, male progeny were only partially fertile. All crosses produced fertile females. Fujioka (1986) also did a preliminary electrophoretic comparison of isozymes in both species, but found no diagnostic loci. The sterility of hybrid males and the distinct X chromosomes of An. freeborni and "southern occidentalis" convinced Barr (1988) that "southern occidentalis" was, indeed, a new species.

Chromosome aberrations

The laboratory strain of An. hermsi that was used by Morrison (1985), Mencheca (1986) and Fujioka (1986) originated from the Malibu region of Orange County, California. No mosquitoes were found to have any inversions or other kinds of aberrations. Mencheca (1986) also studied the polytene chromosomes of the progeny of 25 field caught females from the same region; no aberrations were found.

Methods and Materials

Collections

Larvae and adults of An. freeborni were collected from California, Oregon and Washington during July-October, 1988. In California, many areas throughout the Sacramento Valley were sampled as well as several locations in the foothills of the Sierra Nevada, one location in the coastal

range (Clear Lake), two locations in the Owens Valley, and one location near the coast at Palo Alto (Table 3-1, Fig. 3-2). In Oregon, one area in the northcentral part of the state was sampled as well as one location near the Columbia River. Both collection sites in Washington were along the Yakima River valley. Mosquito larvae, collected in 1987 at artesian wells near the town of Vernal in Uintah County, Utah, were provided by Dan Klins and Steve Romney. Although a cytogenetic study was not done on the field-caught material of this strain, ovarian nurse cell polytene chromosomes of 16 F1 females were examined.

At most sites within California, only adults were collected. Mosquitoes were often found in large numbers under bridges or in culverts and were caught with battery-powered aspirators. Otherwise, dippers were used to collect larvae. Larvae were reared in plastic tubs covered with screened lids and were fed a mixture of guinea pig chow, liver powder and yeast. An attempt was made to collect several hundred individuals from each location in order to have enough material for a cytogenetic survey and an analysis of electrophoretic variability.

Chromosome preparation. The technique used to prepare the polytene chromosomes was generally that described by French et al. (1962). Adult females were bloodfed and held at ca. 27°C for 27-30 hours. The ovaries were then removed in dilute Carnoy's and subsequently transferred to a drop of 75% glacial acetic acid placed on a siliconized cover slip

The ovaries were teased apart with the use of minuten pins and allowed to swell in the acid. A small drop of dilute 2%

Table 3-1. Collection site number, location and habitat for samples of Anopheles freeborni collected in California, Oregon and Washington.

SITE N	LOCATION	HABITAT
	CALIFORNIA	
1	Nevada Co , Wolf Creek & Highway 49	Adults resting under bridge; larvae in small pond with vegetation
2	El Dorado Co , Cameo Carson Rd	Margins of small pond with cattails
3	El Dorado Co , Pleasant Valley Rd	Margins of small pond
4	Sacramento Co , Sloughhouse	Adults resting under bridges
5	Sacramento Co., Folsom	Adults caught biting man
6	Sutter Co., Highway 99 & Nuestley Rd near city of Sacramento	Adults resting under bridge
7	Yolo Co., Capay Valley, Guinda	Adults resting under bridge
8	Yolo Co., Knights Landing	Adults resting in culvert pipe
9	Colusa Co , Hillers Landing	Adults resting under bridge
10	Colusa Co , Highway 20 near Hillless	Adults resting under bridge
11	Sutter Co , east of Yuba City on Butte House Rd	Adults resting under bridge
12	Butte Co., Chico	Larvae
13	Tehama Co , Tehama Gyle Rd	Adults under bridge and larvae among algae in irrigation ditch
14	Glenn County east of Willows on Highway 162	Adults resting under bridge
15	LakeCo , Clear Lake	Adults in abandoned buildings
16	Sonoma Co , Sonoma	Adult under bridge over Hutchins Cr
17	Sacramento Co , Highway 99 N Twin Cities Rd	Adults resting under bridge
18	San Mateo Co Jasper Ridge Preserve, west of Menlo Pk on Sand Hill RH	Larvae in margins of pond and Adults resting in outhouses
19	Kern Co , Onyx Canebrake Creek	Adults resting under bridge
20	Inyo Co Big Pine	Adults in culvert pipe
21	Inyo Co Bishop	Larvae in cattail marsh

Table 3-1 continued

	OREGON	
22	Jefferson Co., Madras	Larvae in irrigation ditch
23	Umatilla Co., Hermiston	Larvae in flooded pasture
	WASHINGTON	
24	Benton Co., Richland	Larvae in irrigation ditch and adults in abandoned building
25	Yakima Co., Yakima	Larvae in marsh fringing pasture
	UTAH	
26	Uintah Co, Utah	Larvae in artesian wells

Lactic-Aceto-Orcein stain was added to the cover slip and mixed thoroughly. The ovaries were allowed to stain for 60-90 seconds before a slide was appressed to the coverslip. The preparation was then gently pressed between two layers of filter paper and viewed under the microscope at various magnifications. Each slide was scanned for several examples of the full complement of chromosomes. Chromosomes, banding patterns and inversions were identified by using the description and map prepared by Faran (1981) (Fig. 3-3), and by using a photographic map of the best polytene chromosome preparations. Furthermore, the Marysville strain (from which the ovarian nurse cell polytene chromosome map had been prepared) was obtained from the Walter Reed Army Institute of Research and used as a standard for comparing banding patterns. Permanent slides were prepared by placing the chromosome preparations on dry ice for a few minutes, popping off the coverslip, de-hydrating with 70-100% ethanol, and adding a drop of euparal as a mounting medium

Figure 3-2. Collection sites in California, Oregon and Washington

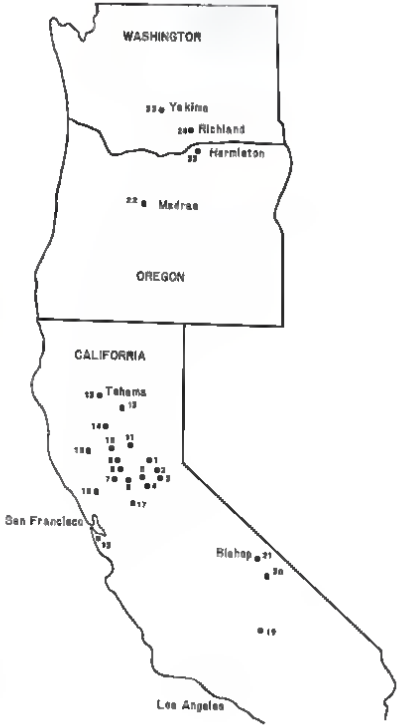

WASHINGTON
Yakima
Richland
Hermiston
Madras
OREGON
CALIFORNIA
Tehama
San Francisco
Bishop
Los Angeles

Figure 3-3. Polytene chromosome map of the ovarian nurse cells (Faran, 1981).

ANOPHELES (ANO) FREEBORNI
OVARIAN NURSE CELL
CHROMSOME MAP

Species Identification

The results of the following cytogenetic study of An. freeborni demonstrated that many populations were actually polymorphic for the "diagnostic" inversion on the X chromosome that supposedly distinguished An. freeborni from An. hermsi. This discovery cast doubt on the use of the inversion on the X chromosome as a reliable character for separating these two sibling species. An independent means of identifying each species was needed.

Concurrent with this investigation, Frank Collins at the Center for Disease Control in Atlanta, Georgia, tested a rDNA probe on three populations of An. freeborni (collected in the Sacramento Valley) and three populations of An. hermsi (collected in southern California). These samples had been collected and sent by Stan Cope of the University of California Los Angeles. The probe was a fragment of rDNA from An. albimanus that had been cloned by Raymond Beach. Frank Collins found that a double digest with restriction enzymes PstI and SalI produced a restriction fragment pattern that was consistently different between the two sibling species; there were no hybrids. This probe, then, appeared to be the only other means of distinguishing An. freeborni from An. hermsi.

The rDNA probe was then employed in order to determine the specific identity of individuals in populations that were polymorphic for In(X)A. At least three individuals from each of 12 collection sites in California, Washington, and

Oregon were sent to Frank Collins in a blind test. These sites included those in which populations of An. freeborni were polymorphic for the inversion on the X chromosome, as well as populations fixed for either homokaryotype. The X chromosomes of most samples sent for rDNA analysis had already been identified by removing the ovaries of the bloodfed females. Samples of mosquitoes from three populations of An. hermsi (collected in southern California by Stan Cope) that had not yet been analyzed with the rDNA probe were also sent.

Statistical Analysis

Some sample sizes were extremely small and not subject to statistical analysis. Otherwise, Pearson Chi-Square, and homogeneity Chi-Square values were calculated by using statistical analysis software (SAS).

Results

Collections

Within the Sacramento Valley, adults were found easily and in large numbers in areas where rice was grown (sites, Table 3-1). In Tehama (site 13), however, larvae were plentiful in irrigated pastureland. In the Owens Valley (sites 20, 21), larvae were collected in a marsh of cattails and adults were found in culverts. Within the foothills of the Sierra Nevada, larvae were collected along the edges of two small ponds, usually within aquatic vegetation. Adults

at Clear Lake (site 15) were found in abandoned buildings near wild rice fields. The only larvae collected during the month of October were found in a pond at Jasper Ridge (site 18). At this site, larvae were found in all four instars and adults were collected from the walls of a nearby restroom. In Yakima, Washington (site 25) and Hermiston, Oregon (site 23) larvae were common in irrigated pasture or in the marshy fringes of these. In Madras (site 22) and Richland (site 24), larvae were found within the aquatic vegetation in irrigation canals. Adults were also collected in Richland within an abandoned building.

Chromosome Variations

Except for differences in inversion frequencies, all populations sampled in this study appeared to have identical polytene chromosome banding patterns, i.e. were homosequential. Certain areas of the autosomes had a propensity for asynapsis, but the degree of asynapsis varied within and between individuals of a given sample: the details are discussed below. Furthermore, all populations, except that collected at Jasper Ridge (site 18), were polymorphic for one or two inversions on chromosome 3. Some populations were also polymorphic for an inversion on the X chromosome. Chromosome 2 had the best band resolution of the complement and was usually asynaptic around the centromere. This asynapsis was not due to any banding differences between homologous chromosomes, but was a result

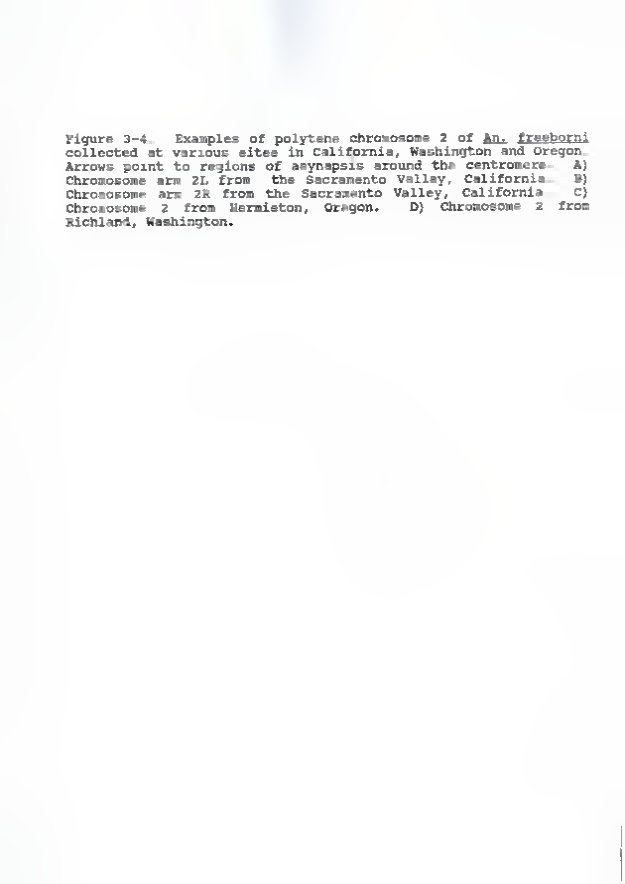
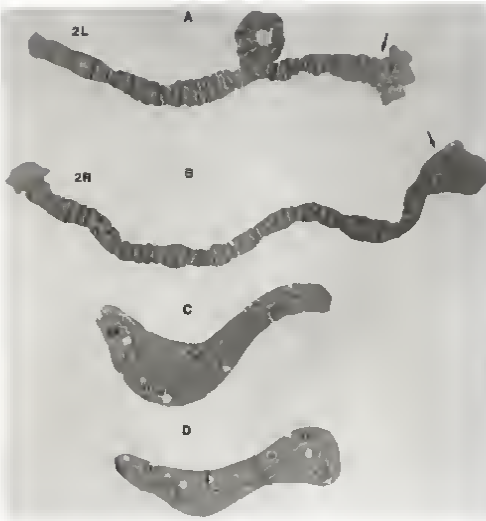


Figure 3-4. Examples of polytene chromosome 2 of An. freeborni collected at various sites in California, Washington and Oregon. Arrows point to regions of asynapsis around the centromere. A) Chromosome arm 2L from the Sacramento Valley, California. B) Chromosome arm 2R from the Sacramento Valley, California. C) Chromosome 2 from Warmiston, Oregon. D) Chromosome 2 from Richland, Washington.

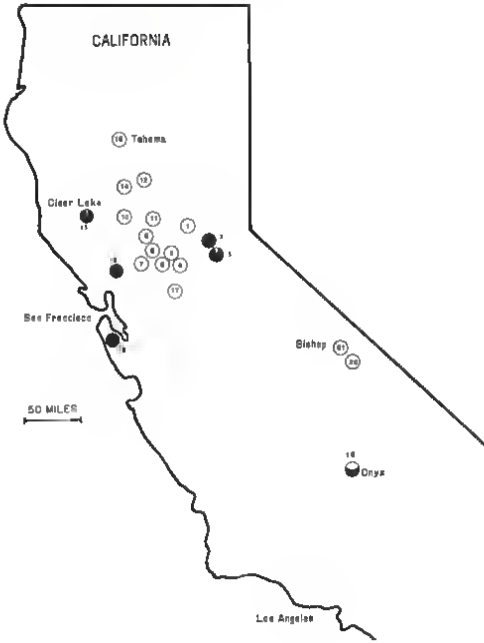

A
2L
B
2R
C
D

of the propensity of the centromeric region to split apart during chromosome preparation (Fig. 3-4). No inversions were found on chromosome 2 in any of the populations sampled.

X Chromosome

All An. freeborni collected in the Sacramento Valley (Table 3-1, Fig 3-2) and in the Owens Valley had the X chromosome described (Kitzmiller and Baker 1963, Faran 1981) as that of An. freeborni (Figs. 3-5, 3-8). Hereafter the X chromosome homokaryotype found in populations within the Sacramento Valley will be referred to as the standard homokaryotype. Populations sampled near the coastal region or the foothills of the Sierra Nevada, however, were either fixed for the inversion homokaryotype (type found in An. hermsi) or were polymorphic for the inversion (included heterokaryotypes). At Clear Lake (site 15), for example, 8% of the mosquitoes sampled were heterokaryoptypes. Since the frequency of the inversion at Clear Lake was 0.98, a very large sample would have been necessary in order to find one or more standard homokaryotype individuals (assuming Hardy-Weinberg equilibrium). At Onyx (site 19) the frequency of heterokaryotypes appeared to be larger, but the sample size was too small at this site to provide an accurate estimate of karyotype frequency. Jeeper Ridge (site 18) and Camino (site 2), on the other hand, were fixed for the inversion karyotype. Another sample taken near

Figure 3-5 Frequency of the standard (White) and inversion (black) karyotype for the X chromosome at various collection sites in California (A), Oregon and Washington (B).


CALIFORNIA
16 Tehama
Clear Lake
San Francisco
Bishop
50 MILES
Onyx
Los Angeles

WASHINGTON
Yakima
Richland
Hermiston
Madras
OREGON
50 MILES

Figure 3-6. Examples of the X chromosome inversion homokaryotype, standard homokaryotype and heterokaryotype from various collection sites in California, Oregon, Washington and Utah. A-B) Standard homokaryotypes from Sacramento, California and Vernal, Utah respectively C) Standard homokaryotype from the Marysville strain of An. freeborni obtained from the Walter Reed Institute of Research; D-E) Inversion homokaryotypes from Camino, California and Richland, Washington, respectively. F) Standard homokaryotype from Richland, Washington. G-I) An inversion homokaryotype from Klamath, Oregon, and two inversion homokaryotypes from Jasper Ridge, California, respectively. J-K) Inversion homokaryotypes from Madras, Oregon and Clear Lake, California, respectively; L-M) Inversion heterokaryotypes from Klamath, Oregon and Richland, Washington.

A
B
C
D
E
F
G
H
I
J
K
L
M

Camino (site 3) was odd in that one standard homokaryotype was found among 12 inversion homokaryotypes.

All mosquitoes collected in Madras, Oregon (site 22) were inversion homokaryotypes (Table 3-2, Fig. 3-5). As one proceeded north into Washington the frequency of the standard homokaryotype increased. At Yakima (site 25), the standard X chromosome reached its highest frequency of 0.90.

Table 3-2. The observed (o) and expected (e) numbers of standard homokaryotypes (S/S) inversion homokaryotypes (I/I) and heterokaryotypes (S/I) for an inversion on the X chromosome of Anopheles freeborni collected from various sites in California, Oregon and Washington.

		Chromosome X								
		S/S		S/I		I/I			Freq	
Site*	n	o	e	o	e	o	e	Chisq	S	I
1	1	1	—	0	•	0	—	•	1.00	0.00
2ab	27	0	0	0	0	27	0	0	0.00	1.00
3ac	13	1	0.08	0	1.08	12	11.08	12.51	0.08	0.92
4	0	9	0	0	0	0	0	0	1.00	0.00
5	2	2		0		0		•	1.00	0.00
6	00	30	00	0	0	0	0	0	1.00	0.00
7	3	3		0	—	0	—	—	1.00	0.00
8	22	22	0	0	0	0	0	0	1.00	0.00
9	35	35	35	0	0	0	0	0	1.00	0.00
10	50	50	00	0	0	0	0	0	1.00	0.00
11	22	22	22	0	0	0	0	0	1.00	0.00
12	27	27	27	0	0	0	0	0	1.00	0.00
13	44	44	44	0	0	0	0	0	1.00	0.00
14	50	50	00	0	0	0	0	0	1.00	0.00
15b	00	0	0.01	5	4.00	95	35.20	0.00	0.04	0.00
16	1	—	—			1			1.00	1.00
17	10	16	16	0	0	0	0	0	1.00	0.00
18a	41	0	0	0	0	41	41	0	1.00	1.00
18	4°	0	•	3		1	—	•	0.88	0.42
20	9	0	9	0	0	0	0	0	1.00	0.00
21	2	2	—	0	—	0	—	—	1.00	0.00
22ab	15	0	0	0	0	15	15	0	0.00	1.00
23c	47	0	3.01	14	10.00	27	24.57	3.00	0.22	0.72
24d	73	26	27.73	38	34.46	8	10.73	0.75	0.62	0.38
25a	00	45	40.00	0	9.00	1	0.00	0.62	0.90	0.10

* Sites followed by the same letter do not differ significantly in frequencies of S/S, S/I and I/I (homogeneity Chi Square P = 0.05)

° Includes two females collected by Stan Cope

The observed frequencies of the homokaryotypes and heterokaryotypes in Hermiston (site 23), Richland (site 24), and Yakima (site 25) did not differ significantly from those expected under Hardy-Weinberg equilibrium (Table 3-2) A test of homogeneity was performed to determine whether the seperate samples were sufficiently uniform to express a common over-all ratio of the three karyotypes. No two samples were found to be homogenous (P = 0.05) in Washington (sites 24, 25) and Oregon (sites 22, 23, Table 3-2).

Chromosome Arm 3L

An inversion on 3L was by far the most common inversion in samples collected in California (Table 3-3, Fig. 3-7). In(3L)A included the 'dot and crescent' landmark (Figs 3-8, 3-9), making it very easy to recognize either homokaryotype. This inversion is the same as that described by Frizzi and DeCarli (1954) and Kitzmiller and Baker (1963). Except for Jeeper Ridge (site 18), Clear Lake (site 15), and several locations with small sample size, the frequency of In(3L)A was remarkably similar throughout California and ranged from 0.19-0.33. None of the observed frequencies of homokaryotypes and heterokaryotypes differed significantly from those expected under Hardy-Weinberg equilibrium (Table 3-3). Furthermore, all samples collected in California, except for Jeeper Ridge (site 18), were homogenous (P = 0.05) (Table 3-3).

Table 3-3. The observed (o) and expected (e) numbers of standard homokaryotypes (S/S), inversion homokaryotypes (I/I) and heterokaryotypes (S/I) for an inversion on chromosome arm 3L of Anopheles freeborni collected from various sites in California, Oregon and Washington.

		Chromosome arm 3L								
		S/S		S/I		I/I			Freq	
Site*	n	o	e	o	e	o	e	Chisq	S	I
1	1	0	-	1	-	0	-	-	0.00	0.00
2a	27	13	12.67	11	11.65	3	2.68	0.09	0.33	0.67
3a	13	8	8.46	5	4.05	0	0.48	0.74	0.69	0.78
4a	7	4	3.56	2	2.86	1	0.57	0.64	0.29	0.71
5	2	1	-	1	-	0	-	-	0.25	0.75
6a	80	35	27.94	12	18.3	8	2.94	5.88	0.24	0.76
7	3	1	-	2	-	0	-	-	0.33	0.67
8a	22	10	10.23	10	0.57	2	2.23	0.05	0.32	0.68
9a	35	23	23.00	11	10.00	1	1.21	0.05	0.19	0.81
10a	80	27	28.92	10	20.16	5	3.90	0.54	0.28	0.72
11a	22	14	13.92	7	7.17	1	0.92	0.01	0.21	0.80
12a	23	15	14.08	8	7.01	2	1.00	1.24	0.22	0.78
13a	44	28	26.26	12	15.44	4	2.27	2.21	0.23	0.77
14a	80	31	32.00	18	16.00	1	2.00	0.78	0.20	0.80
15ab	80	45	44.83	12	12.34	1	0.84	0.04	0.12	0.88
16	-	-	-	-	-	-	-	-	-	-
17a	18	11	8.00	4	8.00	2	1.00	1.78	0.89	0.78
18c	41	41	41.00	0	0	0	0	-	0.00	1.00
19	2	0	-	2	-	0	-	-	0.00	0.00
20a	8	8	6.00	3	2.48	0	0.89	0.36	0.17	0.83
21	2	2	-	0	-	0	-	-	0.00	1.00
22bc	15	15	89.00	0	0	0	0	-	0.00	1.00
23c	66	65	64.06	1	1.00	0	0.01	0.01	0.01	0.99
24c	70	68	69.02	1	0.97	0	0.003	0.00	0.01	0.99
25bc	48	45	45.05	3	2.91	0	0.09	0.05	0.03	0.97

* Sites followed by the same letter do not differ significantly in frequencies of S/S, S/I and I/I (homogeneity Chi Square P = 0.05).

Outside of California, the frequency of In(3L)A was very low (Fig. 3-7). In Oregon and Washington the frequency of In(3L)A was 0.03 or less and all samples were also homogenous (P = 0.05) (Table 3.3).

Chromosome arm 3L had an area near the centromere that was commonly asynaptic in all samples (Figs. 3-8). This area

Table 3-7. Frequency of the standard (black), and inversion (white) karyotype for chromosome 3L at various collection sites in California (A), Oregon and Washington (B).

CALIFORNIA
Tehama
Clear Lake
San Francisco
Bishop
50 MILES
Onyx
Los Angeles

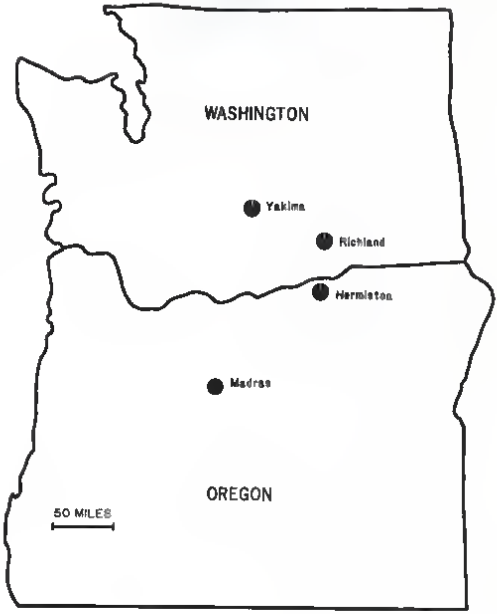
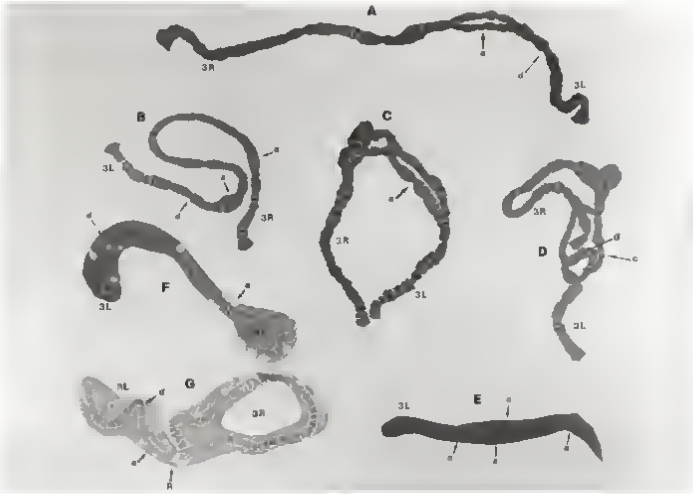

WASHINGTON
Yakima
Richland
Hermiston
Madras
OREGON
50 MILES

Figure 3-8. Examples of chromosome 3 from various collection sites in California, Oregon, Washington and Utah. A-B) Hermiston, Oregon; C) Vernal, Utah showing In(3R)A; D) Sacramento Valley, California showing a double heterokaryotype for In(3R)A and In3L; E) Jasper Ridge, California. F) Hermiston, Oregon; G) Richland, Washington. Arrows point to areas of asynapsis (a), the 'dot and crescent' landmark on 3L (d) and the centromere (c).


A
3R
a
d
3L
B
3L
a
3R
d
F
3L
C
a
3R
3L
D
3R
d
c
3L
G
3L
d
3R
a
E
3L
a
a
a

began at the centromere and extended as far as subzone 35D. The extent to which asynapsis occurred varied within and between individuals and seemed to depend, in part, on the amount of pressure applied to the chromosome preparation. In the inversion homokaryotype, the asynaptic area was transferred to the distal portion of the chromosome. Asynapsis, therefore, did not depend on proximity to the centromere. Furthermore, no banding pattern differences, that might explain this phenomenon, were apparent between homologous chromosomes in the asynaptic area.

Chromosome arm 3R

Although Menchaca (1996) reported three inversions on chromosome arm 3R, In(3R)A was essentially the only inversion found on this arm in this study (Fig. 3-9). In(3R)C was observed in rare instances from samples within the Sacramento Valley, but the frequency was too low to be included in any analysis. Since 3R was the most difficult arm to read because of its length and poor banding resolution, it is possible that In(3R)C is more common than observed in this study.

Chromosome arm 3R was sometimes asynaptic in the region of the centromere (Fig. 3-8, 3-9). The asynapsis, however, was not nearly as extensive as that on 3L and seemed to be the result of centromeric splitting during chromosome preparation.

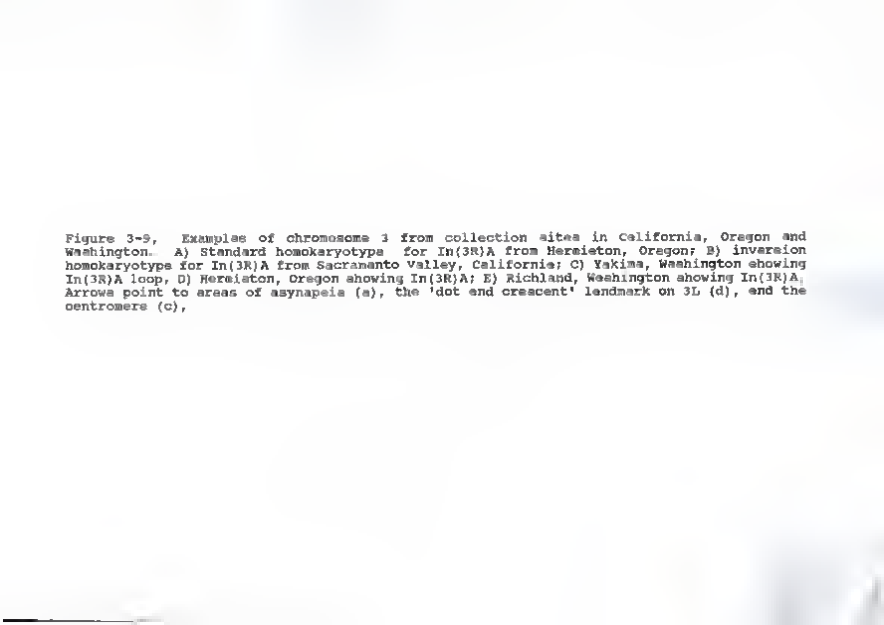
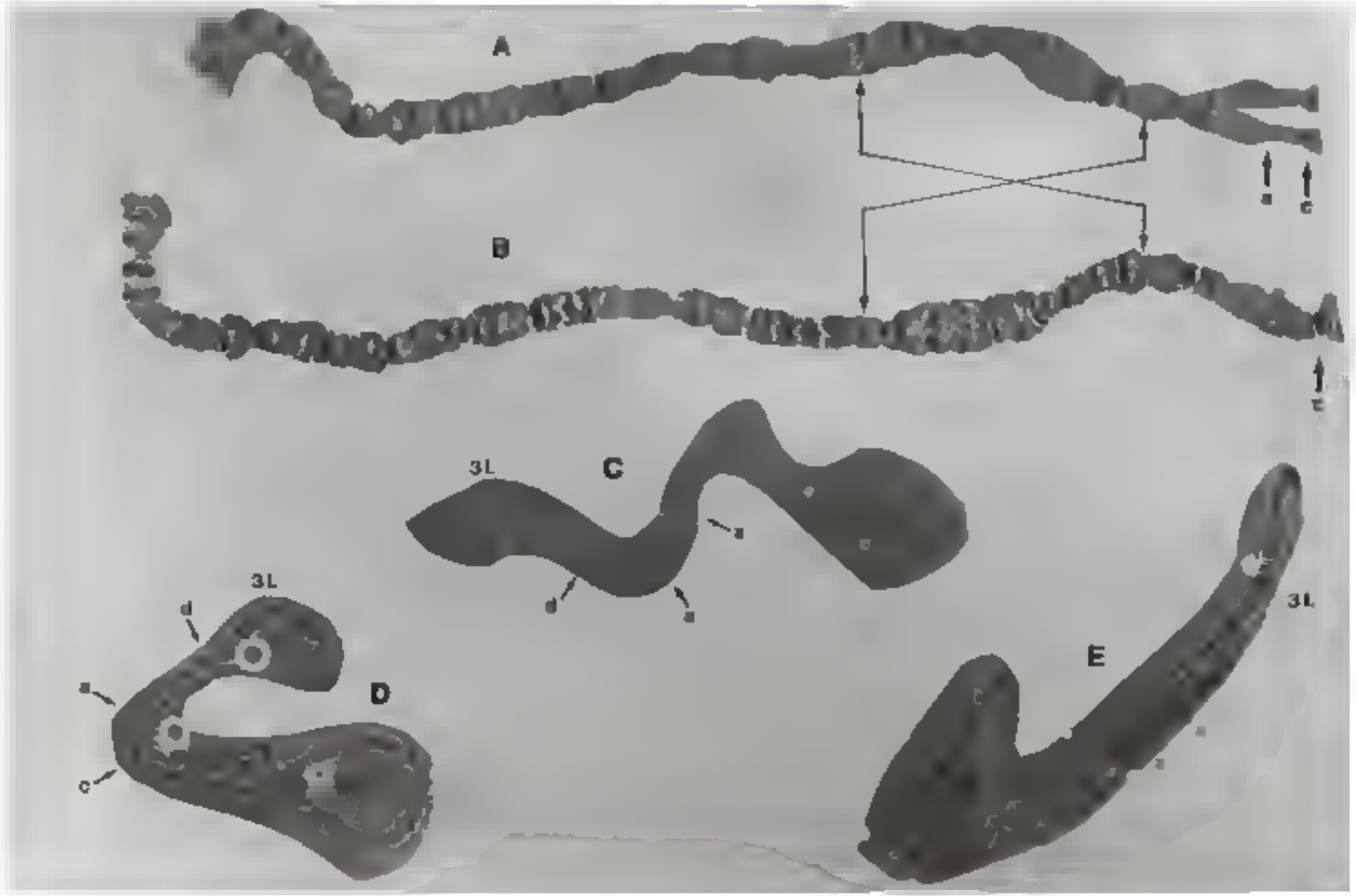


Figure 3-9, Examples of chromosome 3 from collection sites in California, Oregon and Washington. A) Standard homokaryotype for In(3R)A from Hermieton, Oregon; B) inversion homokaryotype for In(3R)A from Sacramento Valley, California; C) Yakima, Washington showing In(3R)A loop, D) Hermieton, Oregon showing In(3R)A; E) Richland, Washington showing In(3R)A. Arrows point to areas of asynapeis (a), the 'dot and crescent' landmark on 3L (d), and the centromere (c),


A
B
C
3L
C
3L
d
D
E
3L

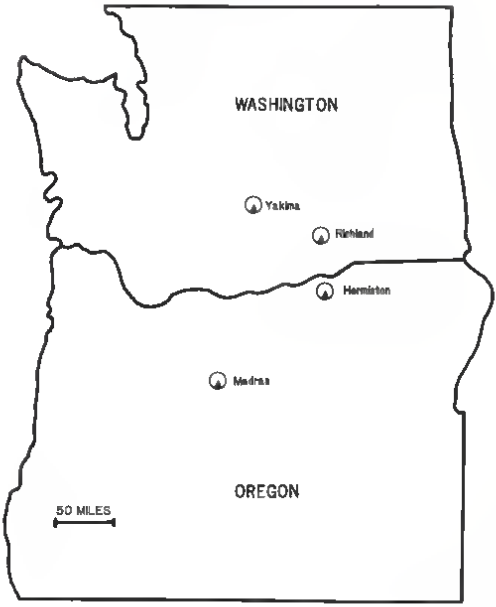
All populations sampled in Oregon (sites 22, 23) and Washington (sites 24, 25) appeared to have similar frequencies of the standard karyotype (Table 3-4, Fig. 3-10). The frequency of the inversion karyotype was low and ranged from 0.09-0.13. The percentage of heterokarytypes ranged from 18.6-26.7 and there were no significant differences between samples based on the homogeneity test ($P = 0.05$).

In California, the reverse was generally true. The inversion was the most common karyotype and the frequency of heterokaryotypes varied dramatically between sites, even when these were in close proximity (Fig. 3-10). At site 10 ($n = 50$), for example, 2% of all individuals sampled were heterokaryotypes, whereas at site 11 ($n = 22$) 30 miles away the frequency was 22.7%. No inversion heterokaryotypes were found in half of the collection sites in which the sample size was at least 13 individuals. These sites appeared to be fixed for the standard or inversion karyotype. Those sites at high elevation or on the coast (sites 2, 3, 15, 18) were fixed for the standard karyotype or had a very low frequency of In(3R)A. Alternatively, in the Sacramento Valley (sites 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 17), populations were fixed for the inversion karyotype or had a high frequency of In(3R)A.

Except for the population at Jasper Ridge (site 18), the percentage of individuals that were heterokaryotypes for In(3R)A and/or In(3L)A ranged from 19.6-66.7 (Table 3-4, Fig 3-11). The percentage of individuals that were

Figure 3-10. Frequency of the standard (white) and inversion (black) karyotype for In(3R)A at various collection sites in California (A), Oregon and Washington (b).

CALIFORNIA
12 Tehama
17
11
Clear Lk
15
16
Camino
2
1
9
7
San Francisco
50 MILES
Los Angeles


WASHINGTON
Yakima
Richland
Hermiston
Madras
OREGON
50 MILES

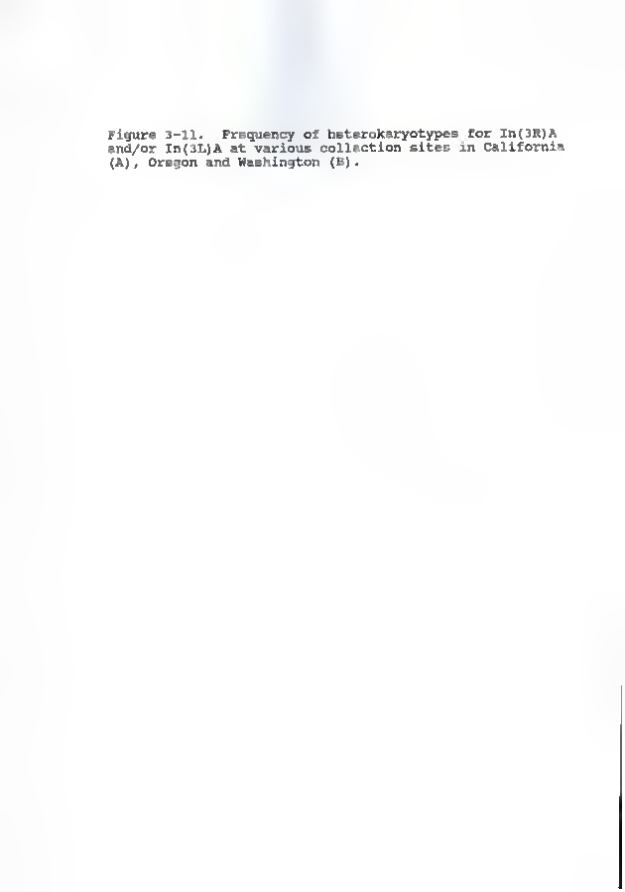


Figure 3-11. Frequency of heterokaryotypes for In(3R)A and/or In(3L)A at various collection sites in California (A), Oregon and Washington (B).

CALIFORNIA
Tahoma
Clear Lake
San Francisco
50 MILES
Los Angeles

WASHINGTON
Yakima
Richland
Hermiston
Madras
OREGON
50 MILES

heterokaryotypes for at least one inversion on any of the chromosomes ranged from 26.1-87.1%. Linkage disequilibrium was tested for those samples with large numbers of individuals and high frequencies of two or more inversions

Table 3-4. Numbers of standard homokaryotypes (S/S), inversion homokaryotypes (I/I) and heterokaryotypes (S/I) for an inversion on chromosome arm 3R of Anopheles freeborni collected from various sites in California. The percentage of heterokaryotypes for In(3R)A and/or In(3L)A is also given.

					Freq		% Heterok. In(3L)A +
Site*	n	S/S	S/I	I/I	S	I	In(3R)A
2a	27	27	0	0	1.00	0.00	40.7
3ab	13	13	0	0	1.00	0.00	38.5
4	6	0	3	3	0.25	0.75	66.7
5	2	0	0	2	0.00	1.00	50.0
6d	49	3	11	35	0.17	0.83	42.9
7	3	0	1	2	0.17	0.83	66.7
8e	22	0	0	22	0.00	1.00	45.5
9e	35	1	3	31	0.07	0.93	40.0
10e	50	0	1	49	0.01	0.99	38.0
11d	22	0	5	17	0.11	0.89	54.5
12a	23	0	2	21	0.04	0.96	31.8
13d	44	0	8	36	0.09	0.91	45.5
14a	50	0	0	50	0.00	1.00	36.0
15a	58	56	2	0	1.00	0.00	20.7
17e	16	0	0	16	0.00	1.00	25.0
18e	41	41	0	0	1.00	0.00	0.0
19	3	2	1	0	0.83	0.17	33.3
20a	9	0	0	9	0.00	1.00	33.3
21	2	0	1	1	0.00	0.75	50.0
22c	15	11	4	0	0.87	0.13	26.7
23c	46	37	9	0	0.90	0.10	19.6
24bc	70	57	13	0	0.91	0.09	21.4
25c	48	37	11	0	0.89	0.11	27.1

* Sites followed by the same letter do not differ significantly in frequencies of S/S, S/I and I/I (homogeneity Chi-Square, P = 0.05).

The association of homokaryotypes and heterokaryotypes of one chromosome or chromosome arm with respect to those on another chromosome or chromosome arm did not differ significantly from random expectations (Tables 3-5, 3-6).

The strain collected from Vernal, Utah, was homosequential to all other strains and had the standard X chromosome arrangement (Fig. 3-6). This strain was also found to have In(3R)A.

Table 3-5. Numbers of observed (o) and expected (e) associations between In(3R)A and In(3L)A inversion systems in samples of Anopheles freeborni. Expected values were calculated from marginal totals.

In(3R)A system		In(3L)A system			
		S/S	S/1	1/1	Totals
SITE 8					
S/S	o	23	10	2	35
	e	22.86	7.84	4.27	
S/1	o	8	1	2	11
	e	7.17	2.46	1.34	
1/1	o	1	0	2	3
	e	1.95	5.57	0.36	
Totals		32	11	8	49
Chi sq = 11.49 (P 0.10)					
SITE 9					
S/S	o	19	11	1	31
	e	20.38	5.49	0.87	
S/1	o	3	5	0	5
	e	1.91	5.89	0.38	
1/1	o	1	5	0	1
	e	0.64	5.30	0.03	
Totals		23	11	1	35
Chi sq = 2.47 (P 0.95)					

Table 3-5 continued

In(3R)A system		In(3L)A system S/S	S/1	1/1	Totals
SITE 11					
S/S	o	8	7	1	17
	e	10.82	5.41	0.77	
S/1	o	5	0	0	5
	e	3.18	1.59	0.22	
1/1	o	0	0	0	0
	e	0	0	0	
Totals		14	7	1	22
Chi-sq = 3.7 (P = 0.70)					
SITE 13					
S/S	o	21	12	3	36
	e	25.09	8.74	2.25	
S/1	o	7	0	1	8
	e	5.67	2.14	0.71	
1/1	o	0	0	0	0
	e	0	0	0	
Totals		28	12	4	44
Chi sq = 5.72 (P = 0.70)					

Table 3-6. Numbers of observed (o) and expected (e) associations between In(3R)A and In(3L)A with In(X)A inversion systems in samples of Anopheles freeborni. Expected values were calculated from marginal totals.

InX system		In(3R)A system S/S	S/1	1/1	Totals
SITE 23					
S/S	o	0	0	5	5
	e	0	0.99	5.99	
S/1	o	0	2	13	15
	e	0	3.00	12.06	

Table 3-6 continued

Ink system		In(3)1A system			
		5/5	5/1	1/1	Totals
1/1	o	0	7	19	26
	e	0	5 26	20.91	
Totals		0	9	37	46
Chi sq = 2.44 (P 0 951					
SITE 25					
5/5	o	0	11	28	39
	e	0	8 04	30.09	
5/1	o	0	0	8	8
	e	0	1 64	6 18	
1/1	o	0	0	1	1
	e	0	0 23	0 78	
Totals		0	11	37	48
Chi sq = 3 26 (P 0 90)					
SITE 24					
5/5	o	0	7	17	24
	e	0	4.80	19 21	
5/1	o	0	6	31	57
	e	0	7.41	29 62	
1/1	o	0	1	8	8
	e	0	1 81	7.22	
Totals		0	14	56	70
Chi sq = 1 94 (P 0.951					
SITE 15		In(3L)A system			
5/5	o	0	0	0	0
	e	0	0	0	0
5/1	o	5	0	0	5
	e	4 04	0 94	0	
1/1	o	42	11	0	53
	e	42.94	10 02	0	
Totals		47	11	0	58
Chi sq = 1 29 (P 0.951					

rDNA Probe

Samples of mosquitoes from 11 of 12 collection sites in California, Washington and Oregon had the same restriction

enzyme fragment pattern regardless of their type of X chromosome (Table 3-7). Mosquitoes from Jasper Ridge (site 18), and those from samples of An. hermsi collected in southern California by Stan Cope, were the only individuals with a restriction fragment pattern specific to An. hermsi. No individuals were found to have a restriction pattern that was a hybrid of that found in An. hermsi and An. freeborni

Table 3-7. Sample site, type of polytene X chromosome, and the rDNA probe determination of species. F = freeborni type; H = hermsi type, HF = heterokaryotype.

SITE	X CHROM.	rDNA PROBE	SITE	X CHROM.	rDNA PROBE
2	H	F	20	F	F
2	H	F	20	F	F
2	H	F	22	H	F
6	F	F	22	H	F
6	F	F	23	HF	F
6	F	F	23	F	F
12	F	F	23	H	F
12	F	F	24	HF	F
12	F	F	24	F	F
13	F	F	24	H	F
13	F	F	25	HF	F
13	F	F	25	F	F
15	HF	-	25	H	F
15	H	F	1003*	H	H
15	H	F	1003	H	H
18	H	H	1063*	H	H
18	H	H	1063	H	H
18	H	H	1063	H	H
18	HB	F	1074*	H	H
19	-	F	1074	H	H
18	-	F	1074	H	H

* Collections of An. hermsi from southern California-
1003: Riverside Co., Rubidoux, Carleon Pk.
1063: Ventura Co., Piru Creek
1074: San Luis Obiepo Co., Santa Margarita

Discussion

In all populations sampled, chromosome 2 was the only autosome that lacked inversions. There are no records of any inversions on chromosome 2 in either the Nearctic or Palearctic maculipennis complex (Baker and Kitzmiller 1964, Baker 1965, Baker and Kitzmiller 1965). Although there are many interspecific rearrangements of the banding patterns on chromosome 2, the absence of inversion polymorphisms in current populations is puzzling. One reason for this apparent lack of polymorphism in chromosome 2 may be that few species in the maculipennis group have actually been investigated in the wild for inversion frequencies. Nevertheless, there does not appear to be any simple correlation between chromosome size, number of interspecific rearrangements, and inversion polymorphisms.

In general, the X chromosomes of anopheline mosquitoes are so distinctive that they provide the best character for distinguishing sibling species (Kitzmiller et al. 1967, Kitzmiller 1977). Relative to its size, the X chromosome has usually undergone a greater amount of rearrangement than the autosomes. In the An. gambiae complex, for example, the X chromosome represents 11% of the total polytene complement length. Yet, five out of ten fixed inversions in this complex are found on the X chromosomes (Coluzzi et al 1979). There are exceptions, however, to the distinctiveness of the X chromosomes. Some species such as An. stephensi Liston and An. farauti Laveran are homosequential, whereas other

species differ in the frequencies of a common floating inversion (Kitzmiller 1977).

Since, according to Barr (1988), An. hermsi and An. freeborni differ by a fixed inversion in the X chromosome, it was startling initially to find many heterokaryotypes for this inversion in so many populations. The large number of heterokaryotypes raises some important questions:

1. Are An. freeborni and An. hermsi really distinct species?
2. Are An. freeborni and An. hermsi hybridizing in sympatric populations?
3. Are one or both species polymorphic for an inversion on the X chrosomsome?
4. Are populations with a polymorphic inversion on the X chromosome a new sibling species?

Samples from Yakima (site 25), Hermiston (site 23) and Richland (site 24) had frequencies of homokaryotypes and heterokaryotypes that were consistent with those expected under Hardy-Weinberg equilibrium. In effect, populations at these locations appeared to be acting randomly with respect to X chromosome type, and there was no indication of the presence of two or more species.

The results of the rDNA probe indicate that all populations, except that found at Jasper Ridge (site 18), are conspecific. It is noteworthy that the population at

Jasper Ridge was the only one that did not have any polymorphic inversions whatsoever. This is also true for the only other wild population of An. hermsi that has ever been sampled for the presence of inversions (Manchaca 1986). Furthermore, Jasper Ridge was the only site found in the month of October to contain larvae at all stages of development. Mosquitoes in the species An. freeborni are generally not found in early larval stages at this late date, but, rather, are adults entering gonotrophic dissociation and migrating to overwintering sites (Bailey and Baerg 1966, Bailey et al. 1972, Washino 1970).

Hybridization of strains from various collection sites (Chapter 3) confirmed the results obtained with the rDNA probe. Mosquitoes from Jasper Ridge (fixed for In(X)A), when crossed to a strain from Sacramento (fixed for the standard X karyotype) or Clear Lake (fixed for In(X)A), produced sterile males. The pattern of sterility was the same as that reported by Fujioka (1986) when he crossed An. hermsi to An. freeborni. However, when the Sacramento strain was crossed to the Clear Lake strain, all progeny were fertile; in effect, the type of X chromosome did not effect fertility.

It is clear from this study that An. hermsi and An. freeborni cannot be distinguished on the basis of the X chromosome. Results from the cytogenetic survey, laboratory hybridizations, and the rDNA restriction pattern study are all consistent in assigning only the population at Jasper

Ridge (site 18) to the species An. hermsi. In fact, there appear to be no detectable differences in any of the polytens chromosomes of these two species. An. hermsi has merely a subset of the variation found in An. freeborni (this conclusion is also substantiated by observations of hybrid polytene chromosomes described in Chapter 4). The absence of any inversions in An. hermsi substantiates a hypothesis that this species arose as an allopatric subpopulation of An. freeborni.

Jasper Ridge (site 18) is a few miles south of San Francisco and is now the northernmost known limit of distribution of An. hermsi. Prior to this study, An. hermsi was known only as far north as Santa Maria, San Luis Obispo County (Barr et al. 1987, Cope et al. 1988), and no further inland than 75 km from the coast. It is now apparent that this species extends up the California coast as far north as San Mateo County, and probably further. Bailey et al (1972) reported collecting An. freeborni near San Pablo Bay and along the Russian River near Healdsburg (Sonoma Co.). Since both sites are near the coast, it is probable that these mosquitoes were actually An. hermsi.

It appears that populations of An. freeborni are of three different types with respect to the X chromosome. Some are fixed for the inversion homokaryotype (Camino = site 2 and Madras = site 22), others are fixed for the standard homokaryotype (the Sacramento Valley = sites 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 17 and Owens Valley = sites 20, 21),

and some are polymorphic (Clear Lake = site 15, Onyx = site 19, Hermiston = site 23, Richland = site 24, and Yakima = site 25). Ethological barriers to mating may certainly exist between these purported conspecific populations, but unless diagnostic characters are found along with sites where two or more mating types are sympatric, it is not possible at present to distinguish these specifically. Except for site 3 (n = 13), there is no indication that any of the populations sampled in this study represent two or more sympatric sibling species. The compliance of inversion frequencies with Hardy-Weinberg expectations, the absence of sterility in the hybrids of geographically-distant populations, and the lack of linkage disequilibrium support the hypothesis that these populations represent a single species. The only unusual collection site, with respect to the X chromosome, was site 3 (e = 13) in the foothills of the Sierra Nevada. At this site, one standard homokaryotype was found among 12 inversion homokaryotypes. The expected frequency of the standard homokaryotype in Hardy-Weinberg equilibrium would be 0.006.

There are wide microgeographical variations in the frequency of In(3R)A in the Sacramento Valley. As Coluzzi et al. (1979) point out, attempts to explain such variations involve testing various hypotheses that are not mutually exclusive: 1. population bottlenecks with subsequent genetic drift; 2. different selective pressures depending on different adult environments and/or larval breeding places

not uniformly distributed in space and/or time; 3. non-random distribution of the chromosomal variants at the adult stage due to different behavioral responses in a heterogenous environment. Although Coluzzi et al. (1979) suggested that non-random distribution of adults was an important contributing factor in their study, this effect would not seem to be important in this investigation. Within the Sacramento Valley, nearly all adults were collected under similar conditions. The collection sites were near irrigated rice fields and adults were obtained from under bridges. In many areas, bridges appeared to offer the only shelter from the sun.

Population bottlenecks with subsequent genetic drift seems a more plausible explanation for the variation in frequency of In(3R)A. Most breeding sites for An. freeborni disappear during the late summer and early fall when rice fields are drained. All or most adult females are then presumed to migrate to overwintering sites within the foothills of the surrounding mountains. These migration flights may be as far as 17.5 miles from breeding sites (Bailey and Beerg 1967, Bailey et al. 1972). How newly-irrigated fields are repopulated with An. freeborni in the spring and summer is not known. Perhaps some females migrate back into the Sacramento Valley, or flooded fields are merely re-populated by the few females that may have overwintered locally. In either scenario, it is possible that newly established populations originate from few

overwintering females.

The great variation in the frequency of In(3R)A between proximate collection sites also suggests that the Sacramento Valley does not harbor one large panmictic population of An. freeborni. Rather, the variation in inversion frequency suggests that An. freeborni does not fly far from its breeding sites in the summer months (Bailey et al. 1972). Since the distribution of rice-growing areas in the Sacramento Valley is patchy, and since suitable breeding sites do not generally exist between them, seasonal (but not permanent) genetic isolation of local populations may be the rule.

Within the Sacramento Valley, In(3R)A is frequent or fixed. At higher elevations, at sites near the coast, or in areas of Washington and Oregon, the standard karyotype is most common. Thus, like the inversion on the X chromosome, the frequency of In(3R)A also changes abruptly with geography.

In(3L)A had a similar frequency in practically all populations and might be maintained by balanced polymorphism. This inversion was also the most common inversion in California. It is curious, therefore, that Smithson (1970) never reported finding In(3L)A in his cytogenetic study of An. freeborni. Smithson (1970) reports frequencies of 0.458-0.484 for heterokaryotypes of In(3R)A in three populations within the Sacramento Valley, including a site near the town of Williams (site 10 in this study).

There are two major problems with the manner in which Smithson (1970) obtained his inversion frequencies. First, Smithson's (1970) chromosome preparations were so poor that he was only able to score the presence or absence of an inversion loop in 6% of his samples; he later abandoned any future studies on the polytene chromosomes of An. freeborni due to his inability to produce readable chromosome preparations. Secondly, Smithson (1970) calculated his inversion heterokaryotype frequencies based on chromosome preparations made from the progeny of field collected females. Since he could not distinguish both homokaryotypes, progeny from matings between two heterokaryotypes would be indistinguishable from those between a heterokaryotype and either homokaryotype. In effect, the progeny of both types of matings would have the same ratio of heterokaryotypes to homokaryotypes.

Due to the poor resolution of his chromosome preparations, it is likely that Smithson (1970) did observe inversion heterokaryotypes for In(3L)A, but incorrectly recorded them as heterokaryotypes of In(3R)A. His estimates of the frequency of In(3R)A heterokaryotypes fall within the range of the combined frequencies of In(3R)A and In(3L)A in this study (Table 3-4, Fig. 3-11).

Polymorphic inversions on chromosome 3 and on the X chromosome are known in other species of anopheline mosquitoes. An. punctipennis Say, An. argyritarsis Robineau-Desvoidy, An. darlingi Root, An. quadriannulatus, and An.

arabiensis Patton, for example, all have inversion polymorphisms on the X chromosome (Kreutzer et al. 1975, Kitzeiller and Baker 1963, Coluzzi et al. 1979). Thus far, all autosomal inversion polymorphisms in the Nearctic and Palearctic An. maculipennis complex have only been found on chromosome 3 (Kitzeiller 1977).

Neither is it particularly unusual for the frequency of an inversion to change throughout the distribution of a given species. Inversion frequency changes and inversion clines are common in drosophilid flies (Da Cunha, et al. 1950, Dobzhansky 1970, Carson 1982) and are also known for species of anopheline mosquitoes (Coluzzi et al. 1979, 1985, Kaiser and Narang pers coe.). Such frequency changes are often correlated with geographic, climatic, and seasonal variations. These correlations suggest that inversion clines are a result (at least in part) of selection acting on the alternative chromosomal arrangements--examples of ecogenetic adjustments within a species. The same conclusion is compelling when the fitness of different homokaryotypes and heterokaryotypes, in laboratory colonies, can be varied by changing environmental conditions such as temperature (Mooe 1955) and crowding (Birch 1955).

In California, the frequency of In(X)A is high or is fixed in areas of higher elevation relative to the Sacramento Valley. In Oregon and Washington, an altitudinal correlation of In(X)A frequency is not as apparent. The

frequency of the inversion increases from Richland (site 24), to Hermiston (site 23), to Madras (site 22), where it appears to be fixed. Likewise, elevation increases from Richland to Madras (Madras is approximately seven times higher than either Hermiston or Richland). Yakima (site 25), however, is twice as high as either Hermiston or Richland, yet has the highest frequency of the standard homokaryotype. Thus, the frequency clines in In(X)A appears to have a latitudinal component as well. Latitudinal clines for inversions have also been found in species A and B of An. quadrimaculatus (Kaiser and Narang pers com.).

Clearly, it would be desirable to make more extensive collections along the foothill areas bordering the Sacramento Valley. In the Sierra Nevada foothills, and at Clear Lake (site 15 within the Coastal Range), populations of An. freeborni are fixed, or nearly so, for the inversion on the X chromosome and the standard arrangement for chromosome arm 3R. In the Sacramento Valley the reverse is true. Is this switch in karyotype sudden, or is there an altitudinal or other environmental cline? The foothills to the Sierra Nevada are gradual and do not appear to represent a formidable isolating barrier. It should be possible, therefore, to find many zones at the border of the Sacramento Valley and the Sierra Nevada that harbor populations of An. freeborni. A cytogenetic study of these populations would help distinguish incipient speciation, ecogenetic adjustment and sibling speciation.

CHAPTER 4

HYBRIDIZATION STUDIES OF ANOPHELES FREEBORNI

Introduction

One means of attempting to arrive at the degree of genetic divergence between populations is through hybridization. If hybridization is not possible, then no genetic flow exists between them. If two populations (or species) hybridize, however, then there are reasonable grounds for inferring that they are closely related or conspecific. Hybrid sterility in anopheline mosquitoes seems to be among the first isolating mechanisms developing during geographic isolation and appears to be a highly reliable guide to the existence of reproductive isolation (Coluzzi 1970). Alternatively, ethological (pre-mating isolation through behavioral incompatability) factors seem to be more important barriers to hybridization in Drosophila (Craddock 1974, Ahearn et al 1974, Carson 1982); many species will hybridize in the laboratory and produce completely fertile offspring. Yet, hybrids are rare or do not occur where these species are sympatric in nature.

Artificial hybridization of different species of anopheline mosquitoes is possible by a technique of forced copulation (Baker et al. 1962). Numerous crosses among the various species of the Palearctic and Nearctic maculipennis

group have been accomplished, and these are summerized by Kitzmiller et al. (1967). Degrees of relatedness are inferred from the relative sterility and viability of progeny produced by the crosses. Further inferences can be made on comparisons of the hybrid polytene chromosomes and the degree of asynapsis. It must be stressed, however, that hybrid sterility and chromosome asynapsis only provide inferences, not absolute proof, on the degree of relatedness between species. At least in anophelines, there does not appear to be any direct relationship between chromosome differentiation by paracentric inversions and genic differentiation. Bullini and Coluzzi (1978) have reported that homosequential species in the maculipennis complex were found to have higher genetic distances than species in the gambiae complex that differed by at least three paracentric fixed inversions. Neither are sterility and asynapsis of chromosomes limited to interspecific crosses, but may also occur among conspecifics. Conspecific crosses of different geographical strains of An. sinensis (Kanda and Ogura 1970, 1972) and of An. stephensi (Rutledge and Ward 1970, Rutledge et al. 1970), for example, can produce differences in fertility and chromosome synapsis. Symbionts (Barr 1980) and transposable elements (Kidwell et al. 1977, Engels 1980) can also produce sterility between conspecifics.

This study reports on the results of crosses between purported conspecific populations of An. freeborni. One aim was to cross two widely separated strains (presumed to be

spatially isolated from each other) that also showed some habitat and chromosomal difference. For this purpose, a strain was obtained from Richland, Washington (WASH strain) and crossed to a strain from the Sacramento Valley, California (Davis strain). The WASH strain was already known to possess a polymorphic inversion on the X chromosome (hitherto unknown in An. freeborni) and was collected in irrigation canals. The DAVIS strain was obtained at a much lower elevation, had no inversion polymorphism in the X chromosome (hereafter referred to as the standard homokaryotype), and generally breeds in rice fields.

Three strains from California, which were fixed for one or the other form of an inversion on the X chromosome, were also crossed. These crosses were deemed necessary when several populations collected in California were found to have relatively high frequencies of inversion heterokaryotypes for an inversion on the X chromosome (Table 3-2, Fig. 3-5). One of the homokaryotypes was that described by Kitzmiller and Baker (1963), Feron (1981) and Menchaca (1986) as being specific to An. freeborni (standard homokaryotype), whereas the other homokaryotype was that described as specific to a new sibling species, An. hermsi (Baker 1965, Morrison 1985, Menchaca 1986, Barr 1988). Thus, the possibility arose that one or both species possessed a polymorphic inversion on the X chromosome, or that these two sibling species were hybridizing in sympatric populations. Alternatively, populations with

heterokaryotypes for InX may have represented another sibling species altogether.

Materials and Methods

The rearing procedures used to maintain all laboratory strains used in this study were generally those described in Chapter 1; deviations from this procedure are specified below. At the time the crosses were done, the WASH strain was in its 9th laboratory generation, whereas the LAKE and JASP strains (see descriptions below) were in the F1 generation. The DAVIS strain had already been maintained in the laboratory for over 4 years.

Prior to crossing the strains, the ovarian polytene chromosomes of at least 10 randomly chosen females from each strain were checked to determine the type of X chromosome present. Pupae from each strain were then sexed according to the length of the hypopygium (Barr 1954), and allowed to emerge as adults in gallon-size tubs with screen lids; sugar pads were provided twice a week.

For crosses between WASH and DAVIS, 50 mosquitoes of each sex were combined in tubs and allowed to mate freely. When the adults were three days old, females were bloodfed on a human arm. Five days post-bloodmeal, twenty females from each cross were transferred to individual plastic vials that were lined with moist filter paper and contained 50 ml water. The number of eggs laid and percentage hatch were recorded for each female. When eggs hatched, two drops of a

food infusion (0.3 gr tetramin fish food added to 0.15 gr yeast in 25 ml water) were added to the vials. Egg batches that did not hatch were held for at least six days (eggs normally hatch within three days) and then dissected and checked for the presence or absence of embryonic development. The spermathecae of females that laid unhatched eggs were dissected for the presence of spermatozoa.

First instar larvae were transferred to enamel pans (18 x 28 cm) containing 100 ml water. The larval diet consisted of three parts guinea pig chow to one part each of hog chow, liver powder and yeast.

Pupae from each tray were counted, separated by sex and allowed to emerge as adults in separate containers/family. After emergence, all adults of a given sex and cross were combined. The testes of at least 10 males from the progeny of each parental cross were dissected and checked for the presence of spermatozoa and normally developed genitalia. All dissections were done in insect saline and the testes were crushed under coverslips to release their contents. Twelve crosses were made in the backcross series including two controls (Table 4-1). The number of eggs/female and percentage hatch were recorded for backcrosses and controls.

The second series of crosses was done between three strains originating from California. One strain was collected at the Jasper Ridge Preserve (JASP strain) just

Table 4-1. Two sets of crosses made between the DAVIS strain (D) and the WASH strain (W) of Anopheles freeborni. The first letter of the backcross hybrids represents the female parent.

EXPERIMENT 1

Parental Crosses

Female		Male
W	X	W
D	X	D
W	X	D
D	X	W

EXPERIMENT 2

Backcrosses and Controls

Female		Male	Female		Male
D	X	D	W	X	W
DW	X	D	WD	X	W
DW	X	W	WD	X	D
DW	X	DW	WD	X	WD
DW	X	WD	WD	X	DW
D	X	DW	W	X	WD
W	X	DW	D	X	WD

West of Palo Alto and ca. 16 km from the coast (Table 3-1, Fig 3-2). This population appeared to be fixed for In(X)A (Table 3-2). Another strain was collected at the north end of Clear Lake (LAKE strain) in Lake County, California. This population was polymorphic for the inversion on the X chromosome (Table 3-2). The third strain was the DAVIS strain.

Pupae and adults were treated in the same manner as described above. At the time these crosses were made, the

LAKE and JASP strains had only been maintained in the laboratory for one generation. Since it was not yet determined whether the JASP, LAKE AND DAVIS strains would mate freely when combined in tube, the parental crosses were done by forced copulation. Soon thereafter, it was determined that all strains would mate freely; thus, the subsequent backcrosses were done without forced copulation. The backcross series included a total of 33 different crosses including 3 controls (Table 4-2). Because there were few mosquitoes in the F1 generation of the JASP and LAKE strains, and because of a lack of space, fewer numbers of females/cross (3-9) were used than in the previous crosses between DAVIS and WASH (9-19).

Ovarian polytene chromosome preparations were made of at least 10 female progeny from each parental cross. These females were bloodfed and held at ca. 27 C for 28 hours. The preparation of the polytene chromosomes was essentially that described by French et al. (1962). Polytene chromosomes were checked for banding pattern homology and the degree of synapsis between homologous chromosomes.

Basic summary statistics of the data were obtained using SAS (Statistical Analysis Software). A one way analysis of variance (ANOVA) was used to compare mean values of the number of eggs laid/female, the percentage of eggs hatched, the number of pupae produced per egg batch, the number of adults emerging, and the sex ratio. The parental crosses,

and the backcrosses and controls were, were analyzed separately by ANOVA.

Table 4-2. Crosses made between the LAKE strain (L), the JASP strain (J) and the DAVIS strain (D) of Anopheles freeborni. The first letter of the backcross hybrids represents the female parent.

EXPERIMENT 1

Parental Crosses

Female		Male	Female		Male
D	X	D	L	X	D
J	X	J	J	X	D
L	X	L	J	X	L
D	X	L	L	X	J
D	X	J			

EXPERIMENT 2

Backcrosses and Controls

Female		Male	Female		Male
D	X	D	D	X	DL
D	X	DJ	D	X	LD
D	X	JD	DL	X	D
DJ	X	D	DL	X	L
DJ	X	DJ	DL	X	DL
DJ	X	J	J	X	J
J	X	DJ	J	X	JL
J	X	JD	J	X	LJ
JD	X	D	JL	X	J
JD	X	JD	JL	X	L
JD	X	J	JL	X	JL
L	X	L	L	X	JL
L	X	LD	L	X	LJ
L	X	DL	LJ	X	L
LD	X	L	LJ	X	J
LD	X	D	LJ	X	LJ
LD	X	LD			

Results

Based on samples of each laboratory strain's polytene chromosomes, it appeared that the JASP, DAVIS, LAKE and WASH strains were homosequential (same banding pattern). The latter three strains, however, were polymorphic for one inversion on 3L (In(3L)A) and one on 3R (In(3R)A) (both described by Frizzi and De Carli 1954, and Kitzeiller and Baker 1963). The WASH and DAVIS laboratory strains had the standard X chromosome karyotype, whereas the LAKE and JASP laboratory strains were fixed for In(X)A. All strains mated freely with each other and produced viable offspring.

DAVIS X WASH Crosses

In the series of parental crosses between DAVIS and WASH, no significant differences were observed in mean egg production, % egg hatch, and % adult emergence (Table 4-3). The sex ratio (males/females) ranged from 1.07-1.48, but there were no significant differences between any of the crosses ($P = 0.05$). Although the two reciprocal crosses differed significantly from each other in the mean number of pupae produced, neither cross differed from the controls.

Backcrosses did not differ significantly from controls in mean number of eggs laid, percentage hatch, and percentage adult emergence. The sex ratio (males/females) ranged from 0.91- 1.36, but none were significantly different ($P = 0.05$).

Table 4-3. Mean number of eggs/female, % hatch, % pupation, and % emergence of adults from crosses between the DAVIS (D) and WASH (W) strains of An. freeborni. Means in same column and experiment, followed by the same letter, are not significantly different (P = 0.05). Separate ANOVAS were done for each experiment (set of crosses).

EXPERIMENT 1

Parental Crosses and Controls

Cross	N	Eggs/ female	% Hatch	% Pupation	% Emergence
W X W	17	131a	79 0a	61.3ab	90 3a
D X D	17	135a	78 9a	64 1ab	92 7a
W X D	19	141a	72 4a	51 9b	92 2a
D X W	18	126a	66.3a	74 1a	92 5a

EXPERIMENT 2

Backcrosses and Controls

Cross	N	Eggs/ female	% Hatch	% Pupation	% Emergence
D X D	10	111ab	95.4a	62.4ef	93 9ab
W X W	8	125ab	96 4a	31 5g	94 9ab
DW X D	10	97b	93 1a	93 1abc	90 3ab
DW X W	10	153a	77 5a	83 2abc	91 3ab
DW X DW	10	129ab	81 4a	81.7abc	90 3ab
D X DW	9	120ab	92.7a	79 5abcd	87 4b
W X DW	10	129ab	90 8a	92 8a	92.2ab
WD X W	9	138ab	90 9a	81 9abc	93.9ab
WD X D	9	148ab	91 0a	93 5abc	96.1ab
WD X WD	10	153a	96 9a	87 0ab	91.3ab
W X WD	10	127ab	84 4a	66 8cdef	92 4ab
D X WD	8	152a	95.7a	75 6abcde	96 7ab
DW X WD	10	93b	93.4a	90 8ab	98 3ab
WD X DW	10	141ab	76 1a	86 9ab	92 6a

Ten of twelve backcrosses produced a significantly higher percentage of pupae than controls; in effect, post-hatch mortality was significantly lower among backcrosses. The controls in the backcross series differed significantly from each other in percentage pupation, although this difference was not evident in the parental crosses.

The testes of the hybrid F1 males were similar in appearance and amount of sperm as those of controls, and all genitalia appeared normal. The chromosomes of F1 hybrids synapsed equally well as those of the controls (Fig. 4-1), and the same inversion heterokaryotypes and homokaryotypes were present. Certain regions on chromosomes 2 and 3 had a propensity to asynapse (see Figs 3-4, 3-8, 3-9, 4-1). On chromosome 2, a region around both sides of the centromere was often split. On chromosome arm 3R, asynapsis sometimes occurred between the centromere and subzone 32E. On 3L, asynapsis sometimes extended from the centromere to subzone 35D. These asynaptic zones, however, were not consistent among or even within one individual. Sometimes different chromosomes from the same preparation showed varying degrees of asynapsis. Furthermore, these regions were equally likely to asynapse in the controls. There did not appear to be any differences in banding pattern between homologous chromosomes in asynaptic regions (eg. duplications or deletions) that could explain the lack of pairing. Apparently, the amount of pressure applied to the chromosome

Figura 4-1. Hybrid polytene chromosomes of progeny from crosses between Wash and Davis strains. A) Chromosome 2, B) chromosome 3; C) X chromosome. Arrows point to centromere (c) and asynaptic regions (a).

A
c
a
B
a
C
c

preparation contributed, in part, to the presence or degree of ssynapais.

Table 4-4. The mean number of eggs laid/fsmale, % hatch, % pupation, and % emergence of adults for crosses made between the DAVIS (D), LAKE (L) and JASP (J) strains of An. freeborni. Means in the same column, followed by the same letter, are not significantly different (P = 0.05). Separate ANOVAS were done for each experiment (set of crosses).

EXPERIMENT 1

Parental Crosses and Controls

Cross	N	Eggs/ Female	% Hatch	% Pupation	% Emergence
D X D	8	144ab	81 3a	73 9a	91 3a
J X J	9	119ab	64 2a	53.8a	87 4a
L X L	3	118ab	79 6a	59.0a	98 6a
D X L	3	93b	65 1a	73 8a	100 0a
D X J	5	159a	93 2a	60.6a	98 3a
L X D	6	156ab	76 4a	76 8a	87 9a
J X D	8	137ab	93 9a	71 2a	85.9a
J X L	8	126ab	82 2a	67 4a	89.8a
L X J	3	163a	67 5a	56 3a	81.8a

EXPERIMENT 2

Backcrosses and Controls

Cross	N	Eggs/ Female	% Hatch	Cross	N	Eggs/ Female	% Hatch
D X D	5	94ab	78.6abcd	J0 X D	6	134a	99.4a
L X L	4	104ab	70 3abcd	J0 X J	6	98ab	62 2cd
J X J	6	100ab	55 1de	L X L0	4	102ab	96 8ab
D X DJ	8	120ab	16 61	L X DL	5	142a	90.1abc
DJ X DJ	6	142a	25.5ef	L0 X L	8	100ab	97.9ab
J X DJ	8	116ab	19 31	L0 X 0	4	118ab	98.5ab
J X LJ	3	64b	38.4e1	L0 X L0	8	117ab	93.8abcd
L X LJ	3	119ab	3 3f	D X DL	6	122ab	98 9ab
LJ X LJ	6	108ab	10.0f	0 X L0	5	98ab	82 1bcd
D X J0	8	104ab	0f	DL X L	5	67b	74 7abcd
J X J0	6	118ab	0f	DL X DL	4	113ab	96 2abc
J0 X J0	4	122ab	0f	DL X D	4	141a	76 8abcd
L X JL	6	99ab	0f	JL X J	4	127ab	56 5e1
J X JL	6	99ab	0f	JL X L	5	105ab	72 7abcd
JL X JL	5	90ab	01	LJ X L	8	78ab	74 6abcd
DJ X D	6	97ab	95 2abc	LJ X J	8	96ab	88 8abc
DJ X J	4	121ab	95 1abc				

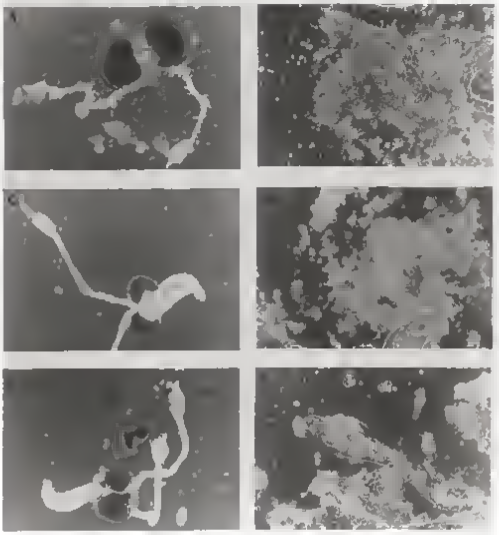
LAKE, DAVIS and JASP Crosses

In the parental crosses between the LAKE, DAVIS, and JASP strains, the controls did not differ significantly from the reciprocal crosses in the mean number of eggs laid/female, in percentage egg hatch, the percentage of individuals pupating, and the percentage of individuals to emergence as adults (Table 4-4). The ratio of females to males was also not significantly different among parental crosses ($P = 0.05$).

All F1 hybrid males from crosses between the LAKE and DAVIS strains had genitalia and quantities of sperm that were similar to those observed in the controls. F1 hybrid males from crosses between LAKE or DAVIS strains with the JASP strain, however, were completely or partially sterile. When the male parent was from the DAVIS or LAKE strain and the female was from the JASP strain, the hybrid male progeny had no sperm in their testes (Fig. 4-2). Although the genitalia appeared to be normal, the testes were often translucent and smaller than the controls. F1 hybrid males, from crosses in which DAVIS or LAKE was the female parent, had varying amounts of sperm in their testes. The amount varied from none to quantities that looked near normal. In general, the testes were filled with what appeared to be globular spermatocytes and partially developed spermatozoa (Fig. 4-2).

The results of the backcross series substantiated the results obtained from the dissection of hybrid males. All

Figure 4-2. A-B) Complete reproductive tract, and squashed testes of hybrid males from crosses between the Lake and Davis strains, C-D) complete reproductive tract, and squashed testes of hybrid males from crosses where the female parent was from the Davis or Lake strains and the male parent was from the Jasp strain; E-F) complete reproductive tract, and squashed testes of hybrid males from crosses where the female parent was from the Jasp strain and the male parent was from the Davis or Lake strains.

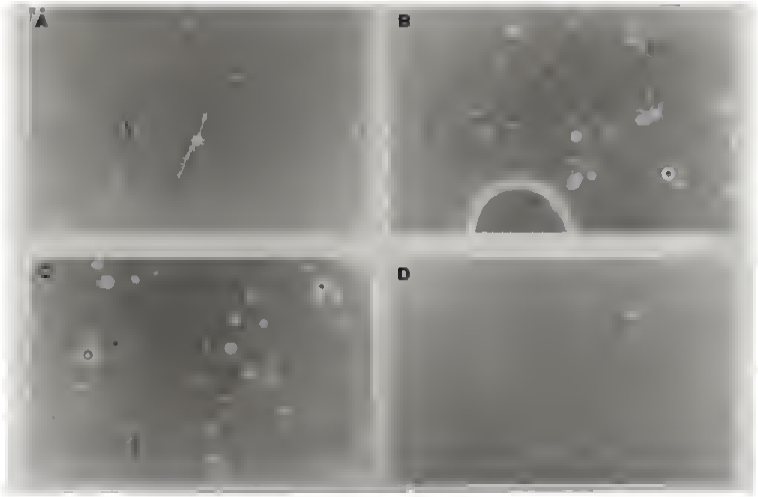


crosses involving hybrid males, in which DAVIS or LAKE had been the parental male, produced eggs that did not hatch (Table 4-4). Nor did the eggs contain any stage of embryonic development. In the reciprocal crosses, the percent hatch was significantly less than the control hatch, and unhatched eggs also contained no embryos.

There were no significant differences between controls and backcrosses for the number of eggs laid/female. All hybrid females were fertile and had a similar percent hatch as controls when backcrossed to either parental strain. Many of the backcrosses had a percent hatch that was higher than the controls, indicating possible heterosis, but none of these differences were significant.

The ovarian polytene chromosomes of hybrid progeny did not differ from controls in the amount or degree of synapsis, or in banding pattern (Fig. 4-3, 4-4, 4-5). Again, chromosomes 2 and 3 had areas with a propensity to be asynaptic in the same zones and in the same manner as described above. The X chromosomes of hybrids between the LAKE and JASP strains synapsed completely, although there was, again, a propensity for the two to be split near the centromere (Fig. 4-5). All hybrids from crosses between JASP or LAKE to DAVIS were heterokaryotypes for In(X)A (Fig. 4-3, 4-4).

Figure 4-3. Hybrid polytene chromosomes of progeny from crosses between Lake and Davis strains. A) Chromosome 2; B) chromosome 3; C-D) inversion heterokaryotypes of X chromosome.


A
B
C
D

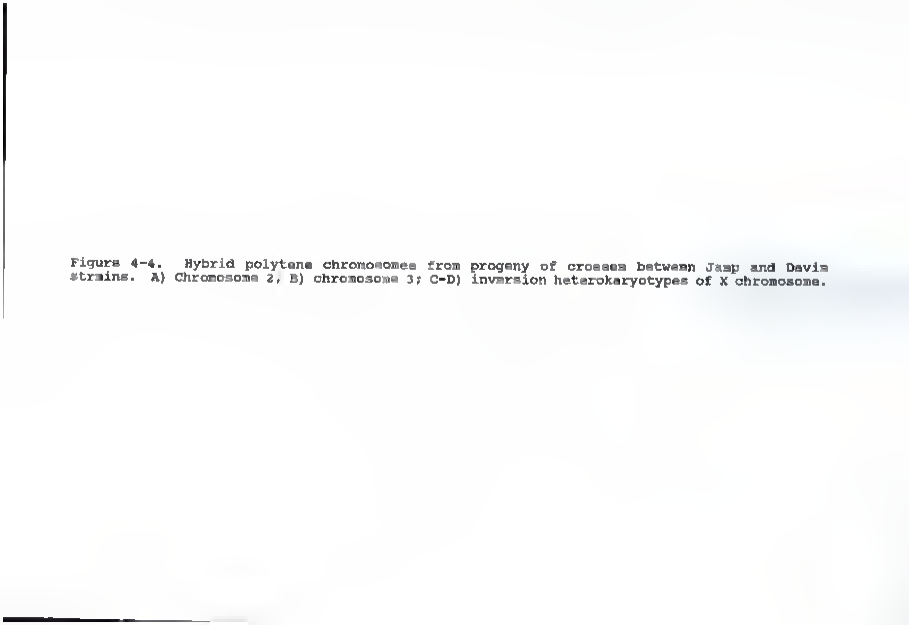


Figure 4-4. Hybrid polytene chromosomes from progeny of crosses between Jasp and Davis strains. A) Chromosome 2, B) chromosome 3; C-D) inversion heterokaryotypes of X chromosome.

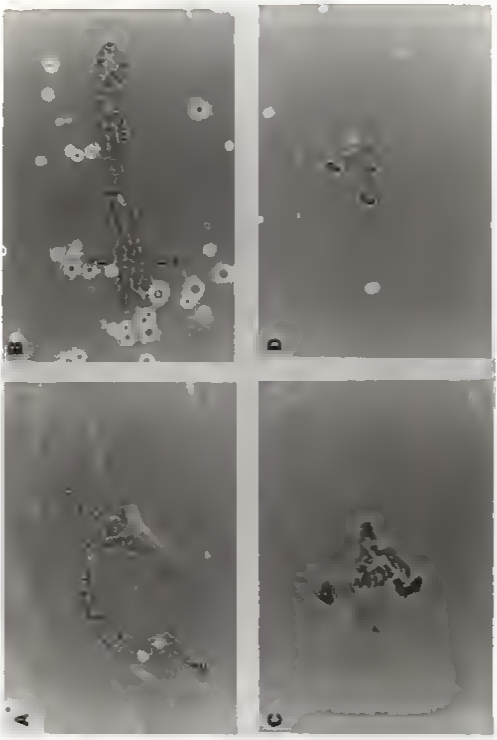
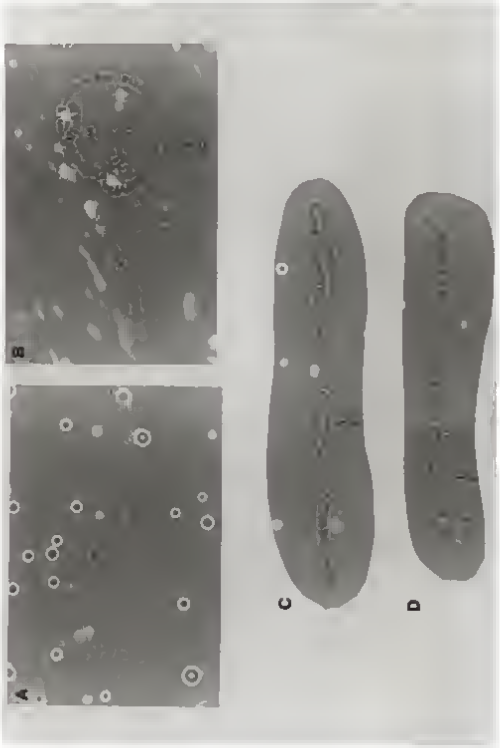

B
D
A
C

Figure 4-5. Hybrid polytene chromosomes from progeny of crosses between Jaap and Lake strains. A) X chromosome; B) chromosome 2; C-D) chromosome 3.


A
B
C
D

Discussion

Unlike many examples among the Drosophila, as yet there are no sibling species of anophelines that, when crossed, do not produce some degree of sterility in the hybrid progeny. This is true even when sibling species are homosequential in polytene banding pattern. An. atroparvus and An. labranchiae, for example, are homosequential species in the Palearctic maculipennis group and produce sterile male hybrids (Bianchi 1968).

The degree and cause of sterility between species varies greatly, but it is generally true that post-zygotic barriers exist between most sibling species of culicids (Kitzmiller et al. 1963?, Kitzmiller 1976). In such crosses, eggs may not hatch, or larvae may only reach a certain instar of development; sex ratios can also be skewed, and adults can be malformed or sterile. In crosses where adults are produced, it is almost always the males that are sterile. Males may be sterile in only one of the two reciprocal parental crosses or in both. Of the 30 possible crosses between six sibling species in the An. gambiae complex, all but two produce sterile males (Davidson 1964, Davidson and Hunt 1973).

In this study, sterile hybrid progeny were produced only when the JASF strain was crossed to either the LAKE or DAVIS strains. Hybrid males were completely sterile in one parental cross and partially sterile in the reciprocal cross. The cause of sterility was due to a complete lack or

small quantity of developed spermatozoa in hybrid males. This pattern of sterility is identical to that found by Fujioka (1988) when he crossed An. hermsi to An. freeborni in both directions.

Cytoplasmic incompatibility between strains due to symbionts (Barr 1980), or the movement of transposable elements do not appear to offer good explanations for the sterility observed in crosses of An. hermsi and An. freeborni. Cytoplasmic incompatibility, as observed in natural populations of Culex pipiens L., is maternally inherited and causes sterility in the parental generation; in effect, the parental female deposits an egg raft that generally fails to hatch.

Transposable elements can cause an effect termed hybrid dysgenesis. Hybrid dysgenesis occurs when an individual from a strain lacking a particular transposon (e.g. P element in Drosophila) is crossed to a male from a strain having the transposon. Therefore, dysgenesis occurs (almost exclusively) in just one of the two reciprocal-cross hybrids. Hybrid dysgenesis is characterized by substantially elevated rates of mutation, chromosomal rearrangement and illicit recombination in males (Drosophila). Dysgenic sterility in Drosophila is usually more pronounced in females (Engels 1980).

In this study, sterility was limited to males and was present in hybrids from both reciprocal crosses. Furthermore, fecundity and fertility of hybrid females was

not significantly different from that of controls, suggesting that massive disruption of germline genetic and developmental integrity (characteristic of hybrid dysgenesis) has not occurred.

With respect to the ovarian nurse-cell polytene chromosomes, the JASP strain is identical to that described for An. hermsi in being fixed for the inversion on the X chromosome (Barr 1988). The JASP strain was also not polymorphic for any inversions (see Chapter 2), which is consistent with the description of An. hermsi by Manchaca (1986). Furthermore, a rDNA analysis (Table 2-7) of the Jasp strain found it to have the same restriction fragment pattern as that of An. hermsi. Finally, the JASP strain was collected as larvae (in all instars) and adults during the month of October. An. freeborni is generally not known to be in early larval stages during this month, but, rather, are adults entering gonotrophic dissociation and migrating to overwintering sites (Bailey and Baerg 1966, Bailey et al. 1972, Washino 1970).

It is clear, then, that the JASP strain is probably An. hermsi and that the X chromosome does not distinguish it from An. freeborni. The LAKE strain, for example, shares the same type of X chromosome with the JASP strain, but hybrid males of these two strains are partially or completely sterile. On the other hand, no sterile progeny are produced when the LAKE and DAVIS strains are crossed,

even though both are fixed for opposite X chromosome karyotypes.

There is no indication from the results of the hybridization experiments and the comparison of the polytene chromosomes that the DAVIS, LAKE and WASH strains represent two or more sibling species. If these three strains are conspecific and if the JASP strain is indeed An. hermsi, then this is the only other known example (apart from An. labranchiae and An. atroparvus) in the culicids of homosequential sibling species.

Are An. hermsi and An. freeborni truly homosequential species? Baker (1965) was the first investigator to compare polytene chromosomes of both species, produce hybrids, and make observations on the hybrid polytene chromosomes. He emphasized the identical banding pattern between An. freeborni and "southern occidentalis", and commented on the complete synapsis of chromosomes in hybrids (except for regions in the centromere areas). Morrison (1985), who prepared a map of the ovarian nurse cell polytene chromosomes of An. hermsi, also noted the great similarity of banding patterns between both species. She stated that there could be some minor banding differences, but acknowledged the difficulty in confirming them due to variable banding expression and resolution in different chromosome preparations. Morrison (1985) made no observations on the hybrid chromosomes of both species. Although Menchaca (1986) stated that the polytene

chromosomes of An. hermsi and An. freeborni were almost identical in banding pattern, she thought there were certain minor differences at the free ends of chromosome 2 and 3. These minor differences were unspecified and not evident in this study or that done by Baker (1965). In addition, Baker (1965) studied specimens of An. hermsi that originated in the same general location as those collected by Menchaca (1986). An example of how two investigators can interpret the banding pattern of the same chromosome differently is illustrated in Figure 4-6. The maps of the X chromosome are so different that they hardly appear to be from the same species. Yet, the samples of An. hermsi studied by both investigators were obtained in Orange County, California (ruling-out variation between distant geographic strains). Perhaps the best "proof" of complete homosequential banding is not the interpretation of banding patterns from photographs or maps (necessarily having a certain subjective element), but a study of synapsis of hybrid chromosomes. In this manner, one can observe both homologues side by side and check asynaptic regions band by band.

Menchaca (1986) observed hybrid polytene chromosomes of both sibling species and stated that there were asynaptic areas in certain zones of chromosomes 2 and 3 that showed band-for-band homology. It is very difficult from her photographs of the hybrid chromosomes, to ascertain the presence and degree of asynapsis mentioned. Those zones where asynapsis is visible without doubt are areas that are

Figura 4-6. A) The salivary gland polytene chromosome map of An. hermsi as described by Manchaca (1986). B) The salivary glandpolytene chromosoma map of An. hermsi as described by Baker and Kitzmiller (1963).

A

B

commonly asynaptic in An. freeborni anyway (see Chapter 3). The other zones she mentions, where asynapsis could be considered minor or questionable, are zones that also show minor asynapsis in An. freeborni. As stated earlier, these particular asynaptic regions varied within and between chromosome preparations suggesting they were, in part, artifacts. The propensity for these regions to split apart during preparation, however, did appear to increase in hybrid polytene chromosome preparations.

In this study, synapsis and asynapsis in hybrid polytene chromosomes was qualitatively no different than that found in the parental lines. Banding patterns appeared to be identical between both species in accordance with Baker (1965). Since Menchaca (1986) found band-for-band homology in the asynaptic areas of hybrid polytene chromosomes (including both free ends of chromosome 2 !), and since all other regions are perfectly synapsed (presumably because their banding patterns are identical), it is probable that An. freeborni and An. hermsi are indeed homosequential.

Finally, Menchaca (1986) stated that the polytene X chromosome of An. hermsi was shorter than that of An. freeborni. She did not, however, mention the amount of variability (standard deviation or variance) observed in her measurements of An. hermsi nor what measurements she took of An. freeborni, if any. Judging from her photographs of polytene X chromosomes obtained from An. hermsi, the variation in size was great. In this study, the X

chromosome of the LAKE strain (An. freeborni with X(In)A) synapsed perfectly with that of the JASP strain (An. hermsi) (Fig. 4-5). If the LAKE strain is An. freeborni, then this complete synapsis demonstrates that both X chromosomes are the same size.

CHAPTER 5

ELECTROPHORETIC ANALYSIS OF ANOPHELES FREEBORNI

Introduction

Studies of soluble enzymatic protein differences controlled by alleles at a single locus (allozymes) can be assayed through electrophoresis and provide information about genetic variation in natural populations. This technique has several desireable features including the simultaneous assay of many enzyme systems per individual and simple mendelian inheritance of electromorphs without dominance at most loci. Electrophoresis, therefore, makes possible the measurement of the amount of genetic differentiation between populations. Geographically isolated populations are subject to various directed and random forces that act to differentiate allele frequencies between them.

The results of enzyme electrophoresis of a single population can be useful in indicating the presence of two or more genetically isolated sibling species (Makale and Richardson 1977). Reproductive isolation of two or more species is suspected when the frequency of heterozygotes for a particular set of alleles is significantly different than that expected under Hardy-Weinberg equilibrium. Thus, enzyme electrophoresis can also uncover diagnostic or

discriminatory loci that are useful in distinguishing sibling species. A locus is defined as diagnostic if an individual can be assigned correctly to one of two species with a probability of 99% or higher (Ayala and Powell 1972). When two populations are sufficiently different at several loci, it is one reason for questioning their conspecificity.

The purpose of this investigation was to study the population genetic structure and divergence of a number of closely and widely separated geographic populations of Anopheles freeborni. Since sibling species are very common in anopheline mosquitoes, this study also attempted to use enzyme electrophoresis to determine whether An. freeborni was actually a species complex.

Materials and Methods

Collection Sites

Specimens of An. freeborni were collected from areas in California, Oregon and Washington during July- October, 1988. In California, many areas throughout the Sacramento Valley were sampled as well as several locations in the coastal range (Clear Lake), two locations in the Owens Valley, and one location near Palo Alto 16 km from the coast (Table 3-1, Fig. 3-2). When larvae were collected, these were reared in plastic tubs covered with screening and were fed a mixture of guinea pig chow, liver powder, hog chow and yeast.

Since not all samples collected above provided large numbers of mosquitoes for enzyme analysis, only a subset of the samples from sites listed in Table 3-1 were used in this electrophoretic study; a few collections of small sample size from sites that were interesting cytogenetically (populations apparently fixed for In(X)A) were also analyzed. The sites chosen for electrophoretic analysis were: site 2 (Camino, CA), site 3 (Pleasant Valley Rd., CA), site 6 (Sutter Co., near city of Sacramento, CA), site 8 (Knights Landing, CA), site 9 (Millers Landing, CA), site 10 (Williams, CA), site 12, (Chico, CA), site 13 (Tehama, CA), site 15 (Clear Lake, CA), site 18 (Jasper Ridge, CA), site 22 (Madras, OR), site 23 (Hermiston, OR), site 24 (Richland, WA), site 25 (Yakima, WA) and site 26 (Uintah Co., Utah). After specimens were collected, observations of ovarian nurse cell polytene chromosomes were made from a sub-sample of females from each location (Chapter 3). The remainder of each female's body and all other material collected were stored at -80°C. Only adult mosquitoes were used in the genetic analysis of field populations.

Electrophoretic Technique

The electrophoretic techniques, enzyme system recipes, and materials employed in this study were basically those described by Steiner and Joslyn (1979). Gels were prepared as 12.5% solutions (w/v) of hydrolyzed starch (Connaught Laboratories, Ontario, Canada) in an appropriate buffer.

An isoline was inbred and selected over several generations to produce a line (DISO line) that was homozygous for most enzyme loci examined in this study. Electromorphs were scored by measuring the distance from where the sample was inserted into the gel to the middle of the electromorph band. Electromorphs of the DISO line were arbitrarily assigned a relative mobility value (Rf value) of 100; these served as control Rf values against which all other electromorphs were compared. Therefore, other electromorphs were assigned Rf values >100 (when the electromorph had migrated further than the control) or <100 (when the electromorph had a slower migration than the control).

Buffers

When employing starch-gel enzyme electrophoresis as a tool for analyzing the genetic structure of an organism, it is necessary to determine which buffer systems are most appropriate for individual enzyme systems used. The first step in this study, therefore, was to compare three different buffer systems (Table 5-1) on a variety of enzymes (Table 5-2) of An. freeborni. A table was then prepared that matched each enzyme with the buffer system that gave the best separation and resolution of electromorphs (Table 5-3).

The three buffer systems that compared were CA-E, Ayala-C, and E.E. (Table 5-1). In most cases the mosquito

samples that were used in this comparison included 4th instar larvae, pupae and adults. In this manner, it was possible to determine the number of zones of activity (possible loci) present for each enzyme system and identify those zones that were restricted to each developmental stage.

Table 5-1. List of buffers and recipes used for electrophoretic analysis.

Buffer System	Buffer Type	Adjusted pH	Chemical Ingredients per Liter H_2O
Ayala-c	Gel	7.0	1.09 g Trizma Base 0.63 g Citric Acid
	Electrode	7.0	16.35 g Trizma Base 9.46 g Citric Acid
CA-8	Gel	6.45	9.00 g Trizma Base 1.90 g Citric Acid
	*Electrode	6.10	166.40 g Trizma Base 66.00 g Citric Acid
5.5	Gel	5.50	2.60 g Trizma Base 1.60 g Citric Acid
	Electrode	5.20	20.25 g Trizma Base 14.64 g Citric Acid

* The cathode buffer chamber received a 1:3 ratio of buffer to H_2O; the anode received a 1:4 ratio of buffer to H_2O.

The next phase of this study was to determine whether the sex, age, developmental stage, and physiological condition of an individual effected the relative movement of electromorphs for a given enzyme locus. For this purpose,

an isoline was inbred and selected over several generations to produce a line (DISO line) that was homozygous for most loci of interest. After choosing the appropriate buffer system from Table 5-1, each enzyme system was tested on individuals from the DISO line that differed in the variables mentioned above. In this manner, it was possible to determine those variables that change electromorph mobility and appearance under specific buffer and enzyme systems.

A third phase of this investigation was to determine the inheritance pattern of various electromorphs at given loci. This was accomplished simultaneously during an attempt to map various loci of An. freeborni. Virgin males and females of the DISO line were force-copulated to virgin males and females of a laboratory strain which originated from a collection made in Sacramento County, California (DAVIS strain). After each female deposited her eggs, she was electrophoresed along with the male parent. When both parents differed in a number of alleles at particular loci of interest, the F1 progeny were reared-through to adults. Virgin progeny of both sexes from these female isolines were then crossed to the homozygous DISO line. Again, both parents from each mating were electrophoresed, and the appropriate F2 families saved and reared to adults. These progeny were then all electrophoresed and analyzed for crossing-over events.

Since different categories of enzymes (e.g. regulatory, non-regulatory and variable-substrate enzymes) can show varying degrees of polymorphism, the choice of enzymes analyzed can bias the amount of variation reported. For example, regulatory enzymes determine pathway rates and are generally moderately polymorphic. Non-regulatory enzymes maintain equilibrium between substrate and product and are least polymorphic. Variable substrate enzymes, such as the esterases, are often very polymorphic. For this reason, enzyme systems from each category were chosen for this electrophoretic study (see Tables 5-2, 5-3).

Statistical Analysis

An updated version of the Biosys-1 computer program developed by Swofford and Selander (1981) was used to analyze allele variation within and between populations. The program computes allele frequencies, measures of genetic variability, deviations of genotype frequencies from Hardy-Weinberg expectations, F-statistics, a variety of similarity and distance coefficients, and constructs dendrograms using cluster analysis and Wagner procedures.

Results

Enzyme Systems and Allozyme Loci

Each enzyme system and its zones of activity (presumptive loci) will be described briefly below. The

zones of activity have, sometimes, been tentatively referred to as loci even though the mode of inheritance and independence of each zone has only been verified for some enzymes. Any effects on the appearance or migration of electromorphs due to mosquito developmental stages or age are also noted; no changes in electromorph mobility were ever observed due to the sex of the mosquito. Male mosquitoes often produced fainter electromorphs than females, but this is probably due to their being much smaller in size than females. It must be kept in mind that the observations that follow are only applicable to the three buffer systems tested in this study.

Table 5-2. List of enzymes, their abbreviations and functional classification. NR = non-regulatory, R = regulatory, VS = variable substrate.

Enzyme	Classification	
ACON	Aconitase	--
ACPH	Acid phosphatase	NR
ADK	Adenylate kinase	R
ADH	Alcohol dehydrogenase	R
ALD	Aldolase	NR
AO	Aldehyde oxidase	R
AKPH	Alkaline phosphatase	NR
CAT	Catalase	NR
EST	Esterase	VS
FUM	Fumerase	NR

Table 5-2 continued

Enzyme	Classification	
G-3PDH	Glyceraldehyde-3-phosphate dehydrogenase	R
G-GPDH	G-Glycerophosphate dehydrogenase	R
G-6-PDH	Glucose-6-phosphate dehydrogenase	R
Glut	Glutamine dehydrogenase	R
GO	Glucose oxidase	--
GOT	Glutamate oxaloacetate transaminase	NR
HAD	Hydroxyacid dehydrogenase	--
HK	Hexokinase	R
IDH	Isocitrate dehydrogenase	NR
LAP	Leucine aminopeptidase	--
LDH	Lactate dehydrogenase	NR
MDH	Malic dehydrogenase	NR
ME	Malic enzyme	R
MPI	Mannose phosphate isomerase	--
ODH	Octanol dehydrogenase	VS
PEP	Peptidase	VS
6PGD	6-Phosphogluconate dehydrogenase	NR
PGI	Phosphoglucose isomerase	R
PGM	Phosphoglucomutase	R
XDH	Xanthine dehydrogenase	R

Table 5.3 Comparison in 3 buffer systems of enzyme systems in larvae (L), pupae (P) and adults (A) of *An. freeborni*. E = excellent (heterozygotes discernable); G = good (heterozygotes discernable, but poor resolution); P = poor (heterozygotes not normally discernable or unsure); Q = bad (bands very faint or unsure). 0 = bands absent or very faint. Enzyme activity is shown for adult mosquitoes only. * = strong activity, + moderate activity, / = weak activity, 0 = absent or very weak

Enzyme Locus	Activ	Buffer System: S-S			CA 8			Ayala-G		
		L	P	A	L	P	A	L	P	A
Acon-1	+	ND	ND	E	ND	ND	E	ND	ND	E
Acph-1	/	G	G	G	E	G	G	G	G	G
Adk 1	+	ND	ND	ND	ND	ND	0	ND	ND	E
Adk 2	+	ND	ND	ND	ND	ND	S	ND	ND	0
Adk 3	+	ND	ND	ND	ND	ND	G	ND	ND	G
Ald 1	0	0	0	0	0	0	0	0	0	0
Ald 1	/	0	0	G	0	G	G	0	G	G
Ap 1	+	ND	ND	0	ND	ND	0	ND	ND	E
Aph 1	G	E	0	0	E	ND	ND	E	E	P
Est 1	*	P	P	P	P	P	P	0	E	0
Est 1	/	ND	ND	G	G	E	G	E	E	0
Est 1	+	ND	ND	ND	0	G	G	0	G	G
Est 2	+	ND	ND	G	G	0	G	0	0	E
Est 3	/	ND	ND	P	G	S	G	E	E	E
Est 4	/	ND	ND	G	E	G	E	E	E	E
Fum-1	G	G	0	G	P	P	P	G	E	G
G 6poh 1	G	ND	ND	ND	ND	ND	ND	G	0	E
G 6poh 1	+	G	G	G	0	0	E	0	0	E
G6pdh-1	0	0	0	0	0	0	0	0	0	0
Glu-1	0	0	0	0	0	0	0	0	0	0
Got-1	/	G	G	0	G	G	P	G	0	0
Got-1	+	ND	ND	ND	G	G	E	G	0	0
Got-2	/	ND	ND	ND	ND	ND	ND	E	P	P
Had 1	+	ND	ND	ND	E	E	S	ND	ND	E
Hk 1	+	ND	ND	ND	G	G	E	ND	ND	E
Hk 2	+	ND	ND	ND	P	P	E	ND	ND	E
Idh-1	+	ND	ND	E	G	E	E	ND	ND	E
Idh 2	+	ND	ND	G	ND	ND	E	ND	ND	0
Lap-1	/	G	0	0	E	G	0	0	G	0
Lap 2	/	G	G	0	0	G	0	G	G	0
Lap 3	/	G	0	0	0	G	0	0	G	0
Lap-4	/	0	P	0	0	P	0	0	P	0
Lap-5	/	P	0	0	P	0	0	P	0	0
Lph 1	/	0	0	P	ND	ND	ND	0	0	P
Lph 2	/	0	0	P	ND	ND	ND	0	0	P
Lph 3	/	0	0	P	ND	ND	ND	0	0	P
Mdh 1	+	ND	ND	ND	E	E	E	E	E	E
Me 1	+	E	E	E	E	E	E	E	E	E
Me 2	+	ND	ND	ND	0	0	G	0	0	E
Mpi 1	+	0	E	E	ND	ND	E	E	E	E
Odh 1	/	E	E	0	E	E	G	E	E	0
Pep-1	/	E	0	0	E	0	0	G	0	0
Pep 3	/	0	E	0	E	E	0	0	ND	0
Pep 3	/	G	P	G	E	P	G	E	P	G
Pep 4	/	ND	ND	ND	P	P	P	P	P	P
Pgm-1	*	ND	ND	ND	E	E	E	E	E	E
6Pgd 1	+	ND	ND	ND	0	0	0	0	P	0
Pgi 1	+	ND	ND	ND	0	0	0	0	E	E
Pgm-1	*	ND	ND	F	P	G	G	G	G	E
Sodh 1	G	E	E	0	E	E	G	E	E	G
Xdh 1	/	ND	ND	P	ND	ND	P	0	0	E

ACON

This enzyme system produces two zones of activity, the slower migrating band being very faint (Fig. 5-1A). Adults, larvae and pupae seem to have similar enzyme activity. Aco can be developed on the same gel slice, without overlap of bands, by the addition of benzaldehyde or other aldehyde as a substrate.

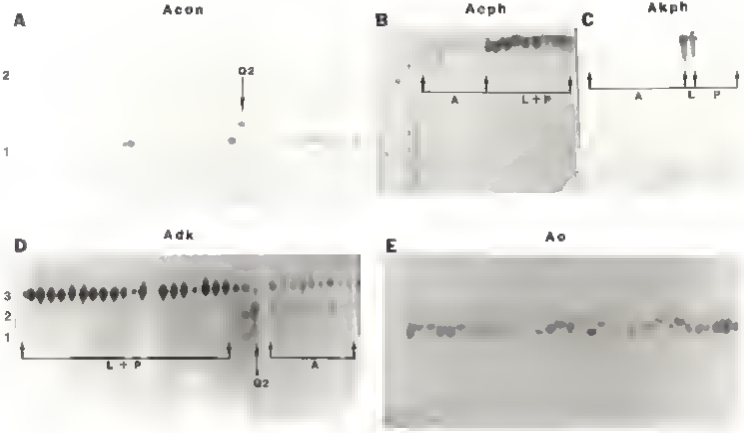
ACPH

This enzyme system has one zone of activity which is best seen on CA-8 buffer. Bands are generally darker in pupae and larvae, and very faint in adults (Fig. 5-1B).

ADK

This system has three zones of activity with good to poor resolution of heterozygotes (Fig. 5-1D). Since the alleles observed in this study have mobility values (Rf values) that are very similar, ADK should be run so that the bands are separated as much as possible (i.e. gel should be run for long time). Adk-1 and Adk-2 show no bands, or very weak ones for larvae and pupae; adults have moderate to weak bands. In Adk-3, however, larvae and pupae have darker bands than adults when CA-8 or 5.5 buffer systems are used. In Ayala-c, the difference in band intensity at Adk-3 between adults, larvae and pupae is minor. To develop this system, the gel slice must be incubated in the staining medium for at least an hour to produce readable bands.

Figure 5-1. Zymograms showing zones of activity for aconitase, acid phosphatase, alkaline phosphatase, adenylate kinase and aldehyde oxidase enzyme systems. A) Acon-1 and Acon-2, the former shows three different electromorphs and monomeric pattern. B) Acph-1 with dark bands in larvae and pupae. C) Akph-1 showing dark bands in larvae only. D) Adk-1-5 showing electromorph intensity differences due to mosquito stage of development. E) Ao-1 showing three banded heterozygotes and both single banded homozygotes. Q2 = control, A = adults, L = larvae, P = pupae.


A
Acon
2
1
Q2
B
Acph
A
L+P
C
Akph
A
L
P
D
Adk
3
2
1
L + P
Q2
A
E
Ao

ADH

No readable bands were detected in any buffer system.

AKPH

This enzyme has a single zone of activity with an extremely slow mobility (Fig. 5-1C). Bands are smeary and generally visible only in larvae.

ALD

Very faint or no readable results were obtained with this enzyme in any buffer system or mosquito stage of development.

AO

This enzyme system produces one zone of activity and is quite polymorphic in An. freeborni (Fig.5-1E). Although the Ayala-c buffer system gives the most readable bands, these are still often smeary or faint. The electromorphs seen on Ao are often found as a contaminants on other gel slices that are being incubated for other enzyme systems (Figs 5-2B, 5-3A). Blood-fed females and old pupae produce darker bands than other stages. Ao can be stained on the same gel slice along with Acon.

CAT

This system produces a single zone of activity with very slow mobility in all three buffer systems (Fig 5-2A). The

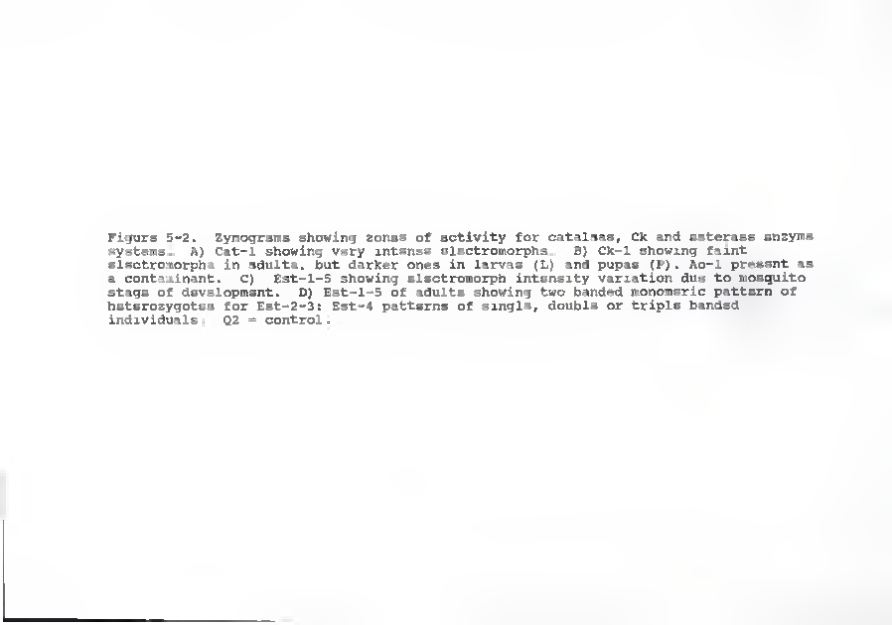
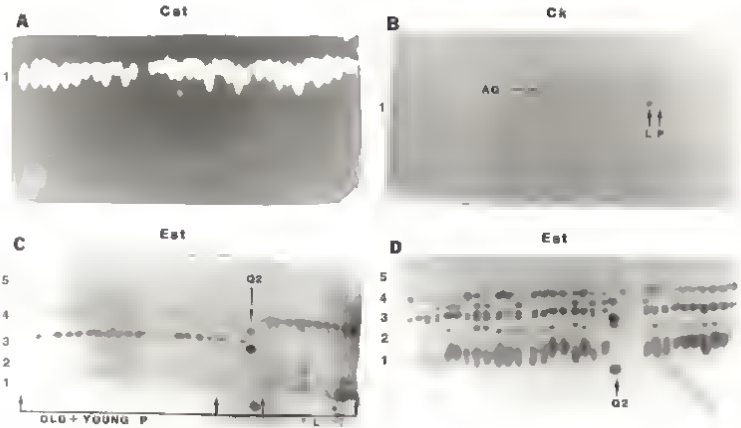


Figure 5-2. Zymograms showing zones of activity for catalase, Ck and esterase enzyme systems. A) Cat-1 showing very intense electromorphs. B) Ck-1 showing faint electromorpha in adults, but darker ones in larvae (L) and pupae (P). Ao-1 present as a contaminant. C) Est-1-5 showing electromorph intensity variation due to mosquito stage of development. D) Est-1-5 of adults showing two banded monomeric pattern of heterozygotes for Est-2-3; Est-4 patterns of single, double or triple banded individuals; Q2 = control.


A
Cat
1
B
Ck
AG
L P
1
C
Est
5
4
3
2
1
Q2
OLD + YOUNG P
L
D
Est
5
4
3
2
1
Q2

best buffer system for this enzyme was found to be CA-8. The activity of this enzyme is so high that bands are very large and smeary. For this reason it is usually not possible to resolve heterozygotes, particularly since the electromorphs have very small Rf value differences. Larvae and pupae produce darker bands than adults.

CK

This enzyme appears to have one zone of activity that is dark in larvae and pupae, but faint in adults (Fig. 5-2B). Bands are generally very blurry.

EST

This enzyme system produces five, possibly six zones of activity (Fig. 5-2C, 5D). The Est-1 locus, the fastest zone in adults, is odd in that individuals can have one, two or three bands. This zone may actually represent two overlapping loci, but this possibility has not been confirmed. The Est-2 locus is slightly slower than Est-1 and has pink bands; larvae and adults have darker electromorphs than do pupae. All other loci have tan to dark brown bands. Est-1, Est-2 and Est-4 appear to be quite polymorphic. Larvae have very dark bands in the Est-4 locus. Est-5 is very faint for all stages of development. There may be another zone of activity that is specific to larvae and pupae and has a mobility intermediate between Est-4 and Est-5. The esterases seem to have a very short

shelf-life, even when kept at -80°C. Thus, if samples are not analysed soon after collection, the esterase loci become unreadable (very faint).

FUM

Very faint or no zones of activity detected with any buffer system.

GLUT

No zones of activity detected for any stage of development.

GO

This enzyme system produces one zone of activity that is very weak in adults but darkly staining in larvae and pupae (Fig. 5-3A). It is not polymorphic and is better resolved in CA-8.

GOT

Two zones of activity are present (Fig. 5-3B). The locus Got-1 can be very polymorphic in some populations, whereas Got-2 has very slow mobility and almost no polymorphism. Adults, larvae and pupae seem to have similar enzyme activity in both loci.

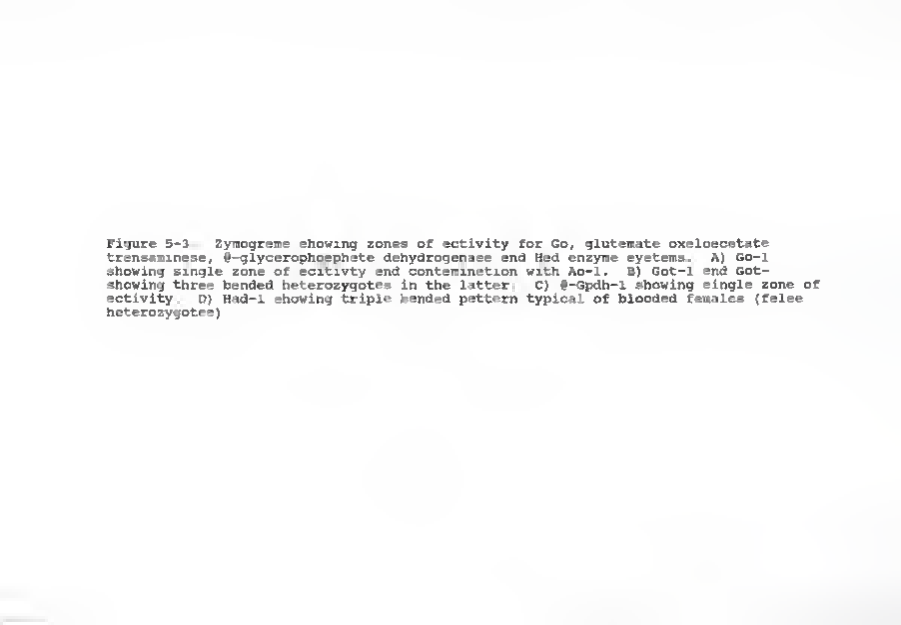
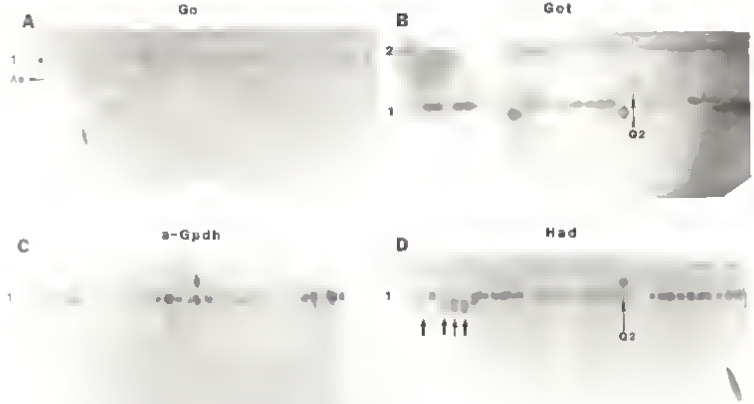


Figure 5-3 Zymogreme showing zones of activity for Go, glutemate oxeloecetate trensaminese, @-glycerophoephete dehydrogenase end Hed enzyme eyetems. A) Go-1 showing single zone of ecitivty end contemination with Ao-1. B) Got-1 end Got- showing three bended heterozygotes in the latter. C) @-Gpdh-1 showing single zone of activity. D) Had-1 showing triple bended pattern typical of blooded females (felee heterozygotes)


A
Go
1
As
B
Got
2
1
Q2
C
a-Gpdh
1
D
Had
1
Q2

6-GPDH

This enzyme system produces one zone of activity with very dark bands and is essentially monomorphic (Fig. 5-3C). Larvae and young pupae have very faint bands, if at all.

G6PDH

No zones of activity detected for any stage of development.

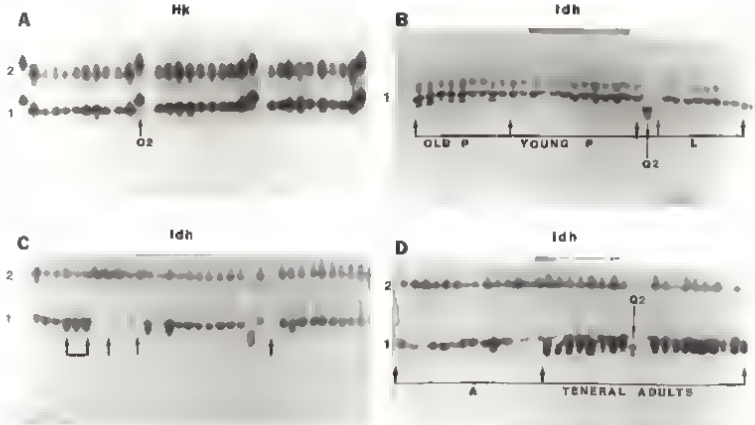
HAD

This enzyme system appears as one locus with slow mobility and little polymorphism (Fig. 5-3D). Females that have had a recent bloodmeal produce bands that are smeary or have a faster Rf value than normal. Sometimes three bands are produced giving the false impression of a heterozygote (false heterozygotes). This enzyme can be stained together with MDH on the same gel slice.

HK

This enzyme system produces two zones of activity with fairly dark bands (Fig. 5-4A). In adults, the electromorphs in the first zone are very dark, but are faint in pupae and larvae. The second zone of activity appears as double bands (perhaps a third locus?). Electromorphs in the second zone of activity are darker in pupae and larvae than they are in adults.

Figure 5-4. Zymograms showing zones of activity for hexokinase and isocitrate dehydrogenase enzyme systems. A) Very dark electromorphs of Hk-1 and H-2. B) Effects of mosquito stage of development on appearance of electromorphs in Idh-1. C) Idh-1 and Idh-2; arrows point to three banded, smeary pattern and blanks typical of blooded females. D) Effects of mosquito stage of development on appearance of electromorphs in Idh-1. O2 = control, L = larvae, P = pupae, A = adult


A
Hk
2
1
O2
B
Idh
1
OLD P
YOUNG P
L
O2
C
Idh
2
1
D
Idh
2
O2
1
A
TENERAL ADULTS

IDH

This enzyme system produces two zones of bands that are very darkly staining (Fig. 5-4B, 4C, 4D). IDH is so active an enzyme that the last slice of any gel will always give readable bands. This enzyme system should be analyzed with CA-8 buffer only, since the locus Idh-2 has an extremely slow migration in 5.5 and Ayala-c buffers. Recently blooded females will produce smeary bands, false heterozygotes or blanks (no band whatsoever) for the Idh-2 locus. In CA-8 buffer, larvae have single bands, pupae have single, double or triple bands (1-2 secondary bands), and adults (less than 24 hrs post-emergence) have triple bands (1-2 secondary bands). In Ayala-c, larvae, pupae and teneral adults have two banded patterns or a very faint third band; larvae and young pupae have fainter bands than do old pupae and adults. Mosquitoes that were more than 7 days old had fainter bands than younger adults in all three buffer systems. For the locus Idh-2, larvae have fainter bands than adults and pupae, but all stages have a single band.

LAP

This enzyme system has five zones of activity (Fig. 5-5A). The first three zones have very fast mobility and only larvae have darkly staining bands. The fourth zone is dark-staining in pupae only and the fifth zone is only visible for larvae. All electromorphs are usually smeary

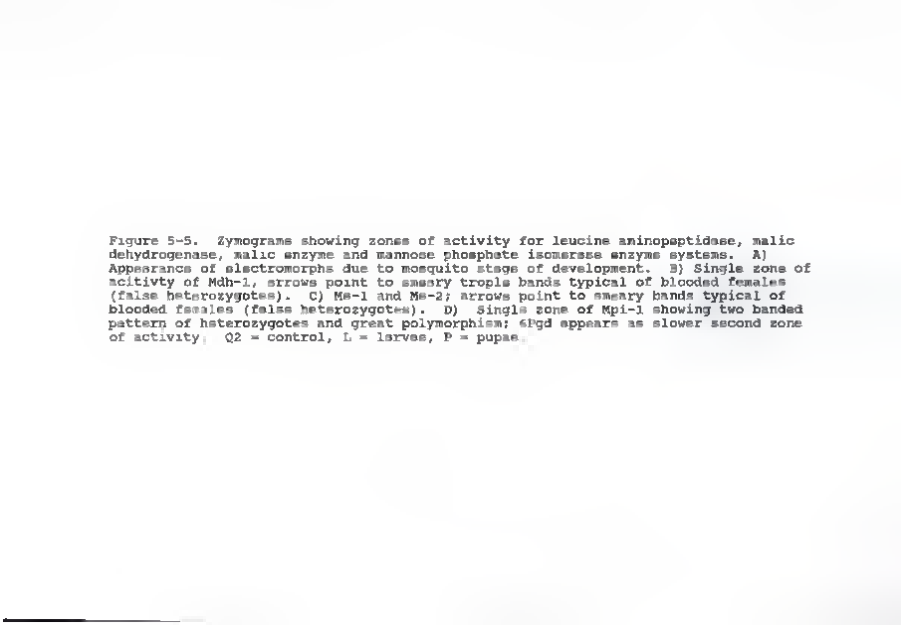
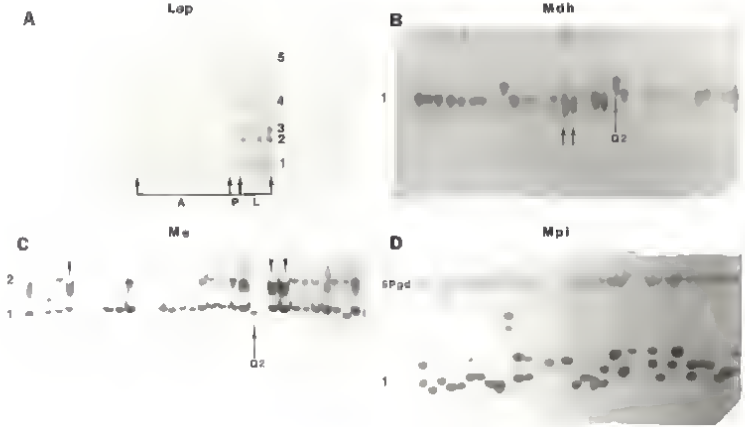


Figure 5-5. Zymograms showing zones of activity for leucine aminopeptidase, malic dehydrogenase, malic enzyme and mannose phosphate isomerase enzyme systems. A) Appearance of electromorphs due to mosquito stage of development. B) Single zone of acitivty of Mdh-1, arrows point to smeary tropic bands typical of blooded females (false heterozygotes). C) Me-1 and Me-2; arrows point to smeary bands typical of blooded females (false heterozygotes). D) Single zone of Mpi-1 showing two banded pattern of heterozygotes and great polymorphism; 6Pgd appears as slower second zone of activity. Q2 = control, L = larvae, P = pupae.


A
Lap
5
4
3
2
1
A
P
L
B
Mdh
1
Q2
C
Me
2
1
Q2
D
Mpi
6Pgd
1

LDH

This enzyme system produced three zones of activity in teneral females and males only (0-24 hrs post-emergence); larvae, pupae and non-teneral adults had no bands.

MDH

This enzyme can be stained together with HAD on the same gel slice (Fig. 5-5B). It has one locus with negligable polymorphism. Blooded females produce smeary bands and false heterozygotes. Larvae, pupae and adults seem to have approximately the same enzyme activity.

ME

This enzyme is very active (produces very dark bands) and can be stained for on the last gel slice (Fig. 5-5C). Two zones of activity are present. Blooded or young females (1-2 days after emergence) produce smeary bands, false heterozygotes or fast Rf values for the Me-2 locus. The Me-2 locus bands are more discrete in Ayala-c and are smeary in CA-8 and 5.5 buffers. In Ayala-c buffer, Me-2 bands have a slightly faster migration of electromorphs in larvae than in old pupae and adults; in 5.5 buffer the reverse is true. The Me-2 locus shows very little polymorphism. Larvae and pupae have fainter bands than do adults.

MPI

Two zones of activity appear in this enzyme system (Fig 5-5D). The Mpi-1 locus has a fast mobility, moderate activity, and its inheritance pattern shows that it is a sex-linked monomeric enzyme. It is also polymorphic, having at least 12 alleles (six of which are shown in Figure 5-5B, 6C). The Mpi-2 locus has a very slow mobility and is the same single zone of activity found on gel slices stained for 6PGD. Furthermore, heterozygotes for the Mpi-2 locus are three banded, suggesting that this enzyme is a dimer. It appears, then, that the Mpi-1 locus electromorphs are actually due to 6PGD. For the Mpi-1 locus, electromorphs of larvae and young pupae are very weak or not visible . Dark pupae (also referred to as 'old'), in which the adult is soon to emerge, will have dark bands on the Mpi-1 locus

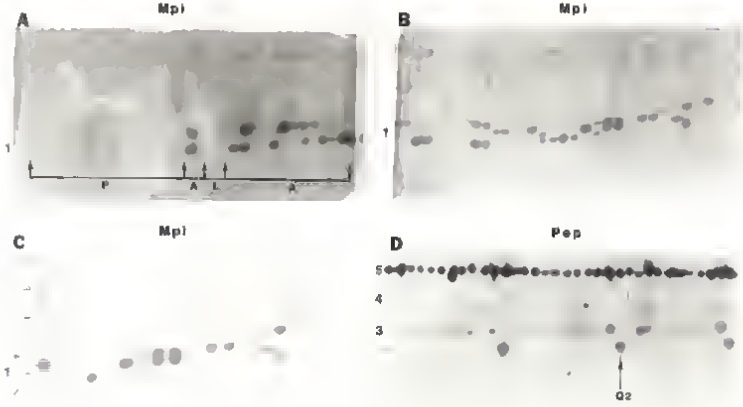
ODH

This enzyme system did not produce readable bands in any buffer system.

PEP

This enzyme system appears to have five zones of activity, two of which are restricted to pupae and larvae. Pep-3 and Pep-5 are usually the only two readable loci; the Pep-4 locus is usually very faint or absent (Fig. 5-6D).

Figure 5-6. Zymograms showing zones of activity for mannose phosphate isomerase and peptidase enzyme systems. A) Appearance of electromorphs due to mosquito stage of development. B) 'Ladder' of different electromorphs in Mpi-1. C) Enlarged view of 'ladder' showing the presence of 7 electromorphs with differernt relative migrations. D) Three zones of activity present in adult mosquitoes for the peptidase enzyme system. Q2 = control, L = larvae, P = pupae, A = adults.


A
Mpl
1
P
A
L
R
B
Mpl
1
C
Mpl
1
D
Pep
5
4
3
Q2

6PGD

This enzyme system produces a zone of activity that is identical to the second zone found on gel slices stained for MPI (Fig.5-7A, 7B). The electromorphs of larvae and pupae are very faint or absent. When Ayala-c buffer is used, blooded females produce smeary bands with faster Rf values (Fig. 5-7A; this effect is not seen in CA-8 buffer.

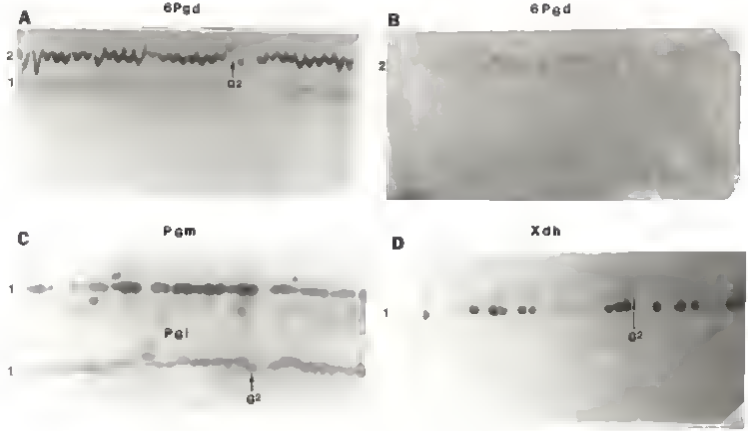
PGI

This enzyme system produces one zone of activity and has a fast mobility (Fig. 5-7C). The gel slice stained for this enzyme must be read immediately after bands appear, since these soon smear and become impossible to score. No electromorph differences are apparent due to sex, physiological conditions, age or developmental stage. This enzyme can be stained for on the same gel slice as that for PGM.

PGM

This enzyme has one clear zone of activity with an area of what appear to be secondary bands (Fig. 5-7C). The Pgm-1 locus is fairly polymorphic and has very dark bands (very active). This enzyme is always possible to read from the last slice of a gel. Adults, pupae and larvae seem to have similar enzyme activity in Ayala-c; in Cs-8 the larvae have very weak activity. This enzyme can be stained together with PGI.

Figure 5-7. Zymograms showing zones of activity for 6-phosphogluconate dehydrogenase, phosphoglucomutase, phosphoglucose isomerase and xanthine dehydrogenase. A) Two zones of activity seen in 6Pgd enzyme system; 6Pgd-2 shows smeary and variously migrating electromorphs typical of blooded females (when Ayala-c buffer system is used). B) Three banded heterozygotes and single banded homozygotes of 6Pgd-2. C) Single zone of activity for Pgi enzyme system and presence of triple banded heterozygote; single zone of activity for Pgm enzyme system showing double banded heterozygotes and four different electromorphs. D) Single zone of activity for Xdh enzyme system. Q2 = control.


A
6Pgd
2
1
G2
B
6Pgd
2
C
Pgm
1
Pgi
1
G2
D
Xdh
1
G2

SODH

Very faint or no zones of activity detected with this enzyme system in any buffer system.

XDH

This enzyme system produces one zone of activity (Fig. 5-7D) that is equally resolved in CA-8 and 5.5 buffer systems. In Ayala-C, bands are a little more blurry than those seen on CA-8 and 5.5 buffer systems.

An analysis of the populations variability of a species through enzyme electrophoresis requires that the particular loci being considered have electromorphs of heterozygotes and homozygotes that are easily distinguished. Some enzyme systems did not produce scorable bands in all the buffer systems employed. Therefore, only enzyme systems that produced bands with clear resolution of homozygotes and heterozygotes were used for genetic analysis--these included a total of 17 enzyme systems (24 presumptive loci) (Table 5-4).

The inheritance patterns of electromorphs for various presumptive loci were determined through crosses as described in the materials and methods section. Two allozyme loci were found to be linked to the X chromosome, Me-1 and Mpi-1. These two loci are also known to be X-linked in Anopheles quadrimaculatus (Lanzaro pers com.).

Table 5-4. Enzyme and buffer systems use in the population genetics analysis of Anopheles freeborni.

BUFFER	
CA-8	AYALA-C
ADK	ACON
GOT	AO
MAD	EST
IDH	α-GPDH
MDH	HK
MPI	ME
6PGD	PGI
PEP	PGM
	XDH

In both species, Me-1 is a tetramer (heterozygote consisting of 5 electromorphs) and Mpi-1 is a monomer (heterozygote has 2 electromorphs) (Table 5-5).

Table 5-5. The maximum number of electromorphs present (in natural populations and genetic crosses of Anopheles freeborni) for heterozygotes of various enzyme loci, and the presumed polymeric enzyme structure. NI = not investigated by crossing studies.

Enzyme	Max. No. Electromorphs Observed		Presumptive
Locus	Nat. Pop.	genetic cross	Structure
Acon-1	2	NI	Monomer
Adk-2	2	2	Monomer
Ao-1	3	3	Dimer
Est-2	2	2	Monomer
Est-3	2	2	Monomer
Est-4	2	2	Monomer

Table 5-5 continued

Enzyme Locus	Max. No. Electromorphs Observed: Nat. Pop.	Max. No. Electromorphs Observed: genetic cross	Presumptive Structure
Got-1	3	3	Dimer
Got-2	3	NI	Dimer
G-Gpdh-1	3	NI	Dimer
Had-1	3	3	Dimer
Hk-1	2	NI	Monomer
Idh-1	3	3	Dimer
Idh-2	3	NI	Dimer
Mdh-1	3	3	Dimer
Me-1	5	5	Tetramer
Me-2	5	NI	Tetramer
Mpi-1	2	2	Monomer
6Pgd-1	3	3	Dimer
Pep-4	3	NI	Dimer
Pgi-1	3	NI	Dimer
Pgm-1	2	2	Monomer
Xdh-1	3	NI	Dimer

Of the 24 locl examined in thie study, the followlng 17 were monomorphic for most populatione (Table 5-6): Adk-1, Adk-2, Adk-3, Got-2, G-Gpdh-1, Had-1, Idh-1, Idh-2, Hk-1, Hk-2, Mdh-1, Me-1, Me-2, Pep-3, Pep-4, Pep-5, 6Pgd-1 and Xdh-1. Consequently, mean heterozygoeity values and the percentage of polymorphic loci (Table 5-7) are relatively low. These values, however, are low estimates because four of the most polymorphic loci observed in thie study (Ao-1, Est-1, Est-3, Est-4) could not be scored reliably and, conaequently, were not included in the enelyeie.

Overall, the populetlone sampled from the Sacramento Velley (eltes 6, 8, 9, 10, 12, 13) hed the highest meen numbere of allelee/locus (1.9-2.8) and the highest percentege of polymorphic loci (20.8-29.2) (Table 5-7). The

samples from Madras, Hermiston, Camino and Pleasant had the lowest amounts of genetic variability, but this may be due to smaller sample sizes available from most of these locations.

Six loci account for practically all polymorphism observed in this study: Acon-1, Est-2, Got-1, Mpi-1, Pgi-1 and Pgm-1. For populations in California, mean heterozygosity values are essentially due to two loci, Mpi-1 and Got-1 (Table 5-8). The Mpi-1 locus was found to have at least 13 alleles, 7 of which are shown in Fig. 5-6. In populations from Washington and Oregon, the Got-1 locus is essentially fixed and mean heterozygosity values are due primarily to polymorphism at the Mpi-1 and Pgm-1 loci.

Frequency clines for particular alleles among populations sampled in California are not apparent. In Oregon and Washington, however, allele A of the Mpi-1 and Pgm-1 loci has an obvious increase in frequency from Madras (southernmost sample site), to Yakima (northernmost site); at the Mpi-1 locus, the frequency of the A allele is low in Madras (ca. 0.04), but is the most common allele in Yakima (freq. A = 0.90) (Table 5-6). This allele is not common among populations in California and is absent from Jasper Ridge (site 18) and Camino (site 2). Outside of Washington, the A allele was common only in the sample collected in Utah (freq. A = 0.52). Several alleles at Mpi-1 that are found among populations in California are not represented in Washington and Oregon (e.g. alleles D, H and J). In

Washington and Oregon, all populations were monomorphic for Est-2 except for Yakima (site 25), Washington. Jasper Ridge was distinguished by being monomorphic for Got-1, Pgm-1 and Est-2, but polymorphic for Pep-4. Although this population is known to be a separate species by other criteria used in this study, no loci were found that were diagnostic or discriminating between it and An. freeborni. This does not rule out the possibility that other enzyme systems will provide diagnostic loci.

Table 5-6. Frequency of alleles and relative mobility values of electromorpha (Rf) at 24 loci for various populations of *Anopheles freeborni*.

		Collection sites														
		Oregon + Washington					Sacramento Valley									
		Bich	Hern	Yak	Nedr	Jasp	Sac	Mitt	Will	Kngts	Tehan.	Chico	Cl Lk	Pleas	Camrn	Utah
Locl	Rf	24	23	25	22	18	6	9	10	8	15	12	15	5	2	26
Acon-1																
(N)		90	33	41	17	49	59	55	58	54	54	41	80	15	80	27
A	100	944	924	951	765	980	924	936	897	980	880	902	875	800	940	852
B	95	050	030	016	176	000	025	027	026	037	019	073	038	087	030	000
C	100	000	030	000	000	020	034	018	034	028	093	024	050	000	000	148
D	89	000	015	008	000	000	017	000	000	057	000	000	000	000	015	000
E	99	000	000	000	000	000	000	000	026	000	000	000	017	033	000	000
F	112	000	000	000	000	000	000	000	000	000	000	000	000	000	000	000
G	95	000	000	025	000	000	000	000	017	000	000	000	000	000	000	000
Adk-1																
(N)		55	44	42	80	36	41	33	36	33	33	22	99	9	22	48
A	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	990
B	109	000	000	000	1.000	000	000	000	000	000	000	000	000	000	000	329
Adk-2																
(N)		49	48	35	15	34	15	34	36	35	31	22	91	8	14	34
A	110	000	000	000	000	000	000	000	000	000	000	000	000	000	000	000
B	91	000	000	.000	000	000	000	000	000	000	000	000	000	000	000	000
C	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
D	84	000	000	.000	.000	.000	000	000	000	000	000	000	000	000	.000	000
E	120	000	000	000	000	.000	000	000	014	000	000	000	000	000	000	000
Adk-3																
(N)		36	44	34	10	33	18	35	36	33	33	22	96	11	10	37
A	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Est-2																
(N)		48	48	45	1	38	41	55	64	23	21	14	48	5	24	35
A	100	000	000	.011	1.000	.934	.915	.891	.945	.955	.000	.929	.615	1.000	.979	.943
B	94	000	.015	.011	000	000	.024	.055	.055	.000	.095	.000	.152	000	.021	.000
C	100	000	000	.011	000	.026	.049	.055	.000	000	000	.000	.233	000	000	000
D	85	000	000	000	000	000	000	000	000	000	000	.036	000	000	000	000
E	80	.010	000	.067	000	.038	000	000	000	000	000	000	000	000	000	.014
F	103	000	000	000	000	000	.012	000	000	000	000	000	000	000	000	000
G	88	000	000	000	000	000	000	000	000	000	000	.036	000	000	000	.043

Table 5-6 continued

		Collection sites														
		Oregon + Washington					Sacramento Valley									
		Rich	Morm	Yak	Nadr	Jasp	Sac	Gill	Vill	Kngts	Tehem	Chico	Cl. Lk	Pleas	Canlon	Utah
Loci	Bf	24	23	25	22	10	6	0	10	8	13	12	10	3	2	26
Mdh-1																
(N)		102	71	76	17	66	60	60	61	60	61	39	60	15	32	63
A	100	.005	.014	.000	.059	.000	.110	.008	.100	.125	.172	.218	.109	.889	.063	.030
B	90	.995	.979	.980	.941	.692	.871	.717	.888	.642	.639	.641	.754	.577	.922	.795
C	88	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	75	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.005
E	81	.010	.007	.020	.000	.269	.194	.159	.231	.808	.189	.141	.141	.038	.016	.169
F	58	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
G	104	.000	.000	.000	.000	.000	.000	.000	.008	.000	.000	.000	.000	.000	.000	.000
H	53	.000	.000	.000	.000	.008	.008	.000	.000	.000	.000	.000	.000	.000	.000	.000
I	88	.000	.000	.000	.000	.015	.012	.017	.015	.000	.000	.000	.016	.000	.000	.000
J	84	.000	.000	.000	.000	.015	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
K	64	.000	.000	.000	.000	.000	.000	.022	.000	.025	.000	.000	.000	.000	.000	.000
Got-2																
(N)		90	84	50	11	77	68	72	58	54	61	80	77	20	60	36
A	100	1.000	1.000	1.000	1.000	1.000	.978	.986	.974	1.000	1.000	1.000	.974	1.000	.983	1.000
B	170	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	154	.000	.000	.000	.000	.000	.015	.014	.000	.000	.000	.000	.000	.000	.018	.000
D	152	.000	.000	.000	.000	.000	.000	.000	.026	.000	.000	.000	.019	.000	.000	.000
E	72	.000	.000	.000	.000	.000	.008	.000	.000	.000						
6-Pgdh-1																
(N)		91	56	35	4	36	37	36	36	30	61	47	65	1	4	71
A	100	1.000	991	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	147	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	121	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	80	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
Mod-1																
(N)		88	48	45	5	63	66	45	33	44	55	43	81	13	21	82
A	100	.994	.990	.974	1.000	1.000	.907	.967	1.000	1.000	.964	1.000	.943	.885	.976	.994
B	140	.000	.000	.011	.000	.000	.022	.022	.000	.000	.000	.000	.025	.115	.024	.006
C	66	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.009	.000	.000	.000
D	131	.000	.010	.011	.000	.000	.000	.000	.000	.000	.000	.000	.014	.000	.000	.000
E	114	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
I	68	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
O	81	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000

Table 5-6 continued

		Collection sites														
		Oregon + Washington					Sacramento Valley									
		Rich	Berm	Yak	Bach	Jeso	Sac.	Bill.	Wilt	Knigh	Tehem.	Chico	Cl Lk	Plesa	Camino	Utah
Locl	Bf	24	23	25	22	16	6	8	10	6	13	12	15	3	2	20
B	170	.000	.000	.000	.000	.000	.211	.000	.000	.000	.000	.000	.010	.000	.000	.000
1	181	.000	.000	.000	.000	.000	.200	.011	.000	.000	.000	.000	.000	.000	.000	.000
Mk_1																
(N)		36	1	25	1	15	15	44	36	35	1	22	46	1	1	45
A	100	1.000	1.000	1.000	1.000	1.000	.967	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	80	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	106	.000	.000	.000	.000	.000	.033	.000	.000	.000	.000	.000	.000	.000	.000	.000
Mk_2																
LN1		37	1	25	1	15	15	44	36	35	1	22	46	1	1	36
A	100	.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	114	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	95	.014	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
Ldh_1																
(N1		97	61	49	17	72	96	33	62	46	65	46	67	13	27	24
A	100	.995	1.000	1.000	1.000	1.000	.000	.989	1.000	1.000	.000	1.000	.976	1.000	.981	1.000
B	120	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.007	.000	.019	.000
C	80	.000	.000	.000	.000	.000	.005	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	120	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
E	165	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	100	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
G	114	.000	.000	.000	.000	.000	.000	.015	.000	.000	.000	.000	.000	.000	.000	.000
H	85	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.015	.000	.000	.000
I	96	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
Ldh_2																
(N1		95	59	89	17	80	100	34	89	46	87	42	72	15	33	34
A	100	.000	1.000	.954	1.000	.988	.971	.989	.988	.989	.969	.976	.986	1.000	1.000	1.000
B	146	.000	.000	.000	.000	.000	.010	.000	.000	.000	.015	.012	.007	.000	.000	.000
C	80	.100	.000	.046	.000	.000	.005	.000	.000	.000	.000	.012	.000	.000	.000	.000
D	81	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
E	165	.000	.000	.000	.000	.015	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	125	.000	.000	.000	.000	.000	.005	.015	.007	.011	.000	.000	.000	.000	.000	.000
Mdh_1																
LN1		96	53	80	5	72	96	51	66	46	58	52	100	15	22	50
A	100	.995	.981	1.000	1.000	.917	.973	.880	1.000	.976	.983	.962	.995	1.000	1.000	.880

Table 5-6 continued

		Collection sites														
		Oregon + Washington					Sacramento Valley									
		Rich	Burn	Yak	Madr	Jasp	Sac	Mill	Wfll	Rngto	Tehma	Chico	Cl Ck	Plema	Canfan	Utah
Locl	Rf	24	23	25	22	18	8	9	10	6	13	12	18	3	2	26
D	111	.000	.000	000	.000	.000	018	.000	.000	.000	000	.000	.000	000	.000	.010
C	83	000	019	000	.000	.000	000	.000	.000	.022	017	.000	.000	000	.000	.000
B	108	.000	000	000	.000	000	000	.000	000	000	000	.000	000	000	.000	000
E	67	.000	000	000	.000	058	000	.000	000	000	000	.000	000	000	.000	.000
F	81	.000	.000	000	.000	.026	000	020	000	000	000	038	000	000	000	000
A	136	.005	.000	000	.000	.000	.000	.000	.000	.000	000	.000	.000	000	.000	.000
Mx-1 (females)																
(N)		67	62	45	4	49	79	19	41	88	88	88	56	5	11	33
A	100	.993	1.000	1 000	1 000	1 000	975	1 000	988	1 000	000	1 000	1 000	1 000	1 000	1 000
B	108	.000	.000	.000	000	.000	013	000	.000	.000	000	.000	.000	000	.000	.000
C	79	000	.000	000	000	.000	008	.000	.000	.000	000	.000	.000	000	.000	.000
D	70	.007	.000	000	000	.000	000	000	.000	000	000	.000	000	000	000	000
B	00	.000	.000	000	000	.000	008	000	.000	000	000	.000	000	000	.000	.000
F	00	000	.000	000	000	000	000	000	000	000	000	.000	000	000	000	.000
D	83	000	.000	.000	000	.000	.000	000	.012	.000	010	.000	.000	000	.000	.000
Mx-1 (males)																
(N)		13	1	15	1	5	1	39	9	00	21	00	1	4	6	8
A	100	000	1 000	1 000	1 000	1 000	1 000	009	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000
B	136	000	.000	.000	000	.000	.000	056	.000	000	000	.000	000	000	.000	000
C	79	077	.000	.000	000	.000	.000	000	.000	000	000	.000	.000	000	.000	.000
D	70	077	.000	000	000	000	000	000	000	000	000	.000	000	000	.000	.000
E	00	000	.000	.000	000	.000	000	000	.000	000	000	.000	000	000	.000	000
F	00	000	000	000	000	.000	000	000	.000	000	000	.000	.000	000	.000	.000
G	89	000	000	000	000	000	000	038	000	000	000	000	000	000	.000	.000
Mx-2 (females)																
(N)		62	00	27	2	29	36	6	11	18	43	10	00	4	13	8
A	100	1 000	1 000	963	1 000	1 000	917	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000
B	150	000	000	000	000	000	028	000	000	000	000	000	000	000	000	000
C	117	000	.000	.000	000	.000	.000	000	.000	000	000	.000	.000	000	.000	000
D	136	000	.000	019	000	.000	.028	000	.000	.000	000	.000	.000	000	.000	.000
E	148	000	000	018	000	000	000	000	000	000	000	000	.000	000	.000	.000
Mx-2 (males)																
(N)		13	1	15	1	3	1	37	9	22	16	14	1	4	4	1
A	100	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000

Table 5-6 continued

		Collection sites													
		Oregon + Washington					Sacramento Valley								
		Eich	Kerm	Yak	Wadr	Jasp	Rec	Will	Will	Kngts	Tehee	Chico	Cl...Lk	Plea...	Camino	Utah
Loci	Ef	24	28	36	32	18	6	9	10	8	13	10	15	3	2	66
Mpi_1 (females)																
101		75	69	55	14	52	76	19	30	13	38	52	80	8	00	52
A	100	605	357	.000	086	.000	.077	132	000	.000	118	045	.000	125	.000	617
B	96	000	000	.000	000	.000	.000	000	000	.000	000	000	.000	000	.000	.000
C	109	000	014	.002	000	010	039	026	063	115	053	001	.037	575	.000	.000
D	115	005	014	.000	000	000	.158	158	131	.000	171	009	.000	000	.000	.000
E	124	224	000	.036	464	423	.516	237	.300	.231	209	159	.353	513	155	.000
F	110	147	100	.036	000	471	245	053	275	269	303	227	404	125	019	183
G	97	011	014	.010	214	000	.044	132	112	.077	055	273	.074	063	.000	.000
H	134	000	000	.000	000	536	.067	000	000	.000	000	000	.015	.000	.000	.000
I	110	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	.000	.000
J	129	000	000	.000	000	010	.000	053	010	.000	000	000	.044	.000	.038	.000
K	00	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	.000	.000
L	59	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	.000	.000
M	00	.005	000	.000	000	000	.026	000	000	.000	013	000	.000	000	.000	.000
Mpi_1 (males)																
101		18	43	30	0	32	24	38	11	00	25	00	41	8	15	15
A	100	556	000	.000	125	000	.000	000	091	.107	087	042	.024	000	000	692
B	00	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	.000	.000
C	109	000	000	.000	125	000	.042	019	091	107	087	083	000	250	533	.000
D	115	000	000	.000	000	000	.292	132	091	143	087	125	.075	000	000	.000
E	124	167	535	.087	425	906	.000	521	091	.000	.217	000	.220	000	333	.000
F	110	222	140	.133	000	438	.292	151	636	.429	174	542	.415	000	067	.000
G	97	056	070	.000	125	000	.083	113	000	.107	217	000	.220	000	000	.000
H	134	000	000	.000	000	031	.000	000	000	.000	000	000	.000	000	000	.000
I	110	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	000	.000
J	129	000	000	.000	000	031	.000	000	000	.018	000	000	.000	250	067	.000
K	00	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	000	.000
L	59	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	000	.000
M	00	000	000	.000	000	000	.000	019	000	.000	087	000	.000	000	000	.000
N	00	000	000	.000	000	000	.000	000	000	.000	043	000	.000	000	000	.000
Gpd_1																
LN1		81	00	60	22	51	67	72	00	47	57	43	101	17	47	62
A	100	994	1.000	1.000	1.000	1.000	.978	972	950	.970	001	1.000	.000	971	1.000	1.000
E	115	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	000	.000
C	87	006	000	.000	000	000	.000	000	000	.000	000	000	.000	000	000	.000
D	100	000	000	.000	000	000	.000	000	000	.000	000	000	.000	000	000	.000

Table 5-6 continued

		Collection sites															
		Oregon + Washington						Sacramento Valley									
		Wish	Herm	Yak	Madr	Josep	Sac...	Mill.	Will...	Knigts	Tehama	Chico	Cl...Lk	Pleas...	Camino	Utah	
Loci	Bf	24	23	25	22	10	8	9	10	8	13	12	15	3	2	26	
B	135	.000	.000	.000	.000	.000	.000	.000	.000	.022	.000	.000	.000	.029	.000	.000	
F	73	.000	.000	.000	.000	.000	.007	.000	.000	.070	.000	.000	.010	.000	.000	.000	
G	127	.000	.000	.000	.000	.000	.015	.014	.050	.000	.000	.000	.005	.000	.000	.000	
H	146	.000	.000	.000	.000	.000	.000	.014	.000	.000	.000	.000	.000	.000	.000	.000	
I	121	.000	.000	.000	.000	.000	.000	.000	.000	.007	.000	.000	.000	.000	.000	.000	
J	67	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
Pop-3																	
(B)		32	50	32	5	35	71	86	48	34	18	15	50	8	16	12	
A	100	1.000	1.000	875	1.000	857	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
B	119	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
C	118	.000	.000	.000	.000	.043	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
D	71	.000	.000	125	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
Pop-4																	
(H)		15	25	7	4	2	60	1	10	1	1	1	13	1	1	18	
A	100	1.000	1.000	1.000	1.000	.750	980	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
B	83	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
C	116	.000	.000	.000	.000	.250	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
H	100	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
Pop-5																	
(I)		81	57	47	4	37	71	32	59	34	32	32	51	9	60	35	
A	100	1.000	1.000	979	1.000	797	1.000	.000	992	1.000	.000	953	980	1.000	1.000	1.000	
H	115	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
C	81	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
G	105	.000	.000	.000	.000	.000	.000	.000	.000	.000	.031	.000	.000	.000	.000	.000	
E	131	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
F	153	.000	.000	.000	.000	122	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
H	59	.000	.000	021	.000	.000	.000	.014	.000	.000	.000	.000	020	.000	.000	.000	
H	36	.000	.000	.000	.000	.000	.000	.000	.008	.000	.000	.000	.000	.000	.000	.000	
I	100	.000	.000	.000	.000	081	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	
J	140	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.045	.000	.000	.000	.000	
Pri-1																	
(A)		100	52	77	23	59	87	73	88	69	61	50	78	18	40	88	
A	100	947	952	962	1.000	1.000	963	952	962	920	975	970	942	947	925	1.000	
B	67	.000	011	019	.000	.000	.007	.007	015	.014	.008	010	013	.026	063	.000	
C	117	.000	.000	.000	.000	.000	.000	.000	.000	.014	.000	.000	.000	.000	.000	.000	

Table 5-6 continued

		Collection sites														
		Oregon + Washington					Sacramento Valley									
		Rich	Mono	Yak	Madr	Josp	Sac	Bill	Mill	Kngts	Tehma	Chico	Cl Lk	Plcns	Camino	Utah
Loci	Nf	24	23	25	22	18	4	8	10	8	13	12	15	3	2	26
B	94	053	038	026	000	000	050	034	023	091	016	020	032	026	013	000
E	111	000	000	000	000	000	000	000	000	000	000	000	013	000	000	000
F	77	000	000	014	000	000	000	000	000	000	000	000	000	000	000	000
Pgm-1																
(N)		108	56	78	21	59	73	79	66	69	65	49	77	15	41	75
A	100	786	688	801	619	983	870	756	879	775	857	796	885	800	988	627
B	124	188	268	173	381	017	041	038	000	087	024	081	026	100	073	393
C	107	000	000	000	000	000	000	000	000	000	000	000	000	000	000	000
D	78	000	027	026	000	000	062	121	061	116	105	153	078	000	000	000
E	81	000	000	000	000	000	007	000	015	000	000	010	000	000	000	000
F	117	000	000	000	000	000	000	000	000	000	000	000	000	000	000	000
G	59	000	000	000	000	000	000	000	000	007	000	000	000	000	000	000
H	159	000	000	000	000	000	000	014	000	000	000	010	000	000	000	000
I	144	019	018	000	000	000	000	000	023	000	000	010	000	000	000	000
J	113	000	000	000	000	000	000	000	015	000	000	000	000	000	000	000
K	70	000	000	000	000	000	000	000	000	000	000	000	000	000	012	000
Xdh-1																
(N)		1	14	9	4	1	9	22	25	25	21	11	18	4	5	1
A	100	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000	909	1 000	1 000	1 000	1 000
B	100	000	000	000	000	000	000	000	000	000	000	000	000	000	000	000
C	81	000	000	000	000	000	000	000	000	000	000	091	000	000	000	000

Table 5-7 Genetic variability at 24 loci. Percentage of polymorphic loci, mean heterozygosity, mean number of alleles per locus, and mean sample size per locus for populations of Anopheles freeborni. Standard errors are in parenthesis.

	Population	Mean sample size per locus	Mean no. of alleles per locus	Percentage of loci polymorphic*	Mean heterozygosity	
					Direct count	Nei's expected**
1	RICHLAND WA	69.7 (6.2)	1.8 (.2)	20.8	.060 (.027)	.059 (.027)
2	BROADBENT, OR	43.6 (4.0)	1.8 (.3)	12.5	.060 (.032)	.060 (.031)
3	YAKIMA, WA	46.4 (4.2)	2.0 (.3)	20.8	.043 (.016)	.059 (.018)
4	MADRAS, OR	10.3 (1.6)	1.3 (.2)	16.7	.056 (.031)	.087 (.036)
5	JASPER CA	44.5 (4.6)	1.8 (.3)	25.0	.081 (.031)	.093 (.030)
6	Sacramento, CA	52.6 (3.8)	2.8 (.4)	29.2	.060 (.037)	.106 (.030)
7	WILLIAMS (...CA	41.3 (4.2)	2.3 (.3)	20.8	.068 (.035)	.094 (.030)
8	WILLIAMS CA	48.6 (4.1)	2.2 (.4)	25.0	.069 (.039)	.069 (.039)
9	KNIGHTS (...CA	39.1 (3.6)	1.9 (.3)	25.0	.100 (.044)	.094 (.040)
10	TEHAMA CA	41.8 (4.4)	2.1 (.3)	20.8	.060 (.037)	.093 (.050)
11	CHICO, CA	30.1 (3.0)	2.0 (.3)	25.0	.069 (.035)	.106 (.040)
12	CLEAR LAKE, CA	65.7 (4.7)	2.3 (.4)	29.2	.069 (.032)	.095 (.035)
13	PLEASANT, CA	9.5 (1.2)	1.3 (.2)	25.0	.087 (.029)	.082 (.037)
14	CAMINO CA	25.8 (2.9)	1.7 (.2)	20.8	.041 (.016)	.048 (.018)
15	UCDAVIS, UT	46.8 (4.9)	1.5 (.2)	20.8	.065 (.026)	.064 (.027)

* A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95

** Biased estimate see NEI (1978)

Table 5-8. Heterozygosity values for individual polymorphic loci in Anopheles freeborni

	Collection sites														
	Bac	Will	Will	Knght	Tehama	Chico	Cl Lk	Plesa	Camino	Rich	Herm	Yak	Modr	Jasp	Utah
Locus	6	9	10	8	15	12	15	3	2	24	23	25	22	18	26
Acph-1															
1/1	59	35	60	54	54	41	60	15	39	90	33	61	17	60	27
Het	.165	.122	.193	.206	.241	.160	.228	.184	.000	.175	.144	.005	.361	.000	.252
Est-2															
1/1	41	55	64	23	21	14	46	5	24	49	60	45	1	38	35
Het	.160	.200	.109	.122	.172	.195	.311	.000	.061	.020	.025	.165	.000	.128	.160
Got-1															
1/1	65	69	65	60	61	39	96	13	32	140	71	76	17	65	83
Het	.099	.451	.510	.529	.526	.522	.429	.518	.166	.029	.042	.039	.111	.448	.338
Mpi-1															
1/1	49	19	80	13	38	40	48	8	26	95	35	60	14	32	52
Het	.805	.612	.791	.756	.776	.796	.699	.727	.538	.561	.612	.187	.686	.595	.000
Pgi-1															
1/1	67	73	80	69	61	60	78	10	60	168	52	77	25	60	83
Het	.372	.079	.074	.159	.000	.039	.111	.101	.140	.101	.000	.112	.000	.000	.000
Pgm-1															
1/1	73	70	60	69	63	60	77	15	41	100	60	78	21	60	75
Het	.235	.364	.223	.378	.294	.347	.213	.180	.000	.343	.455	.327	.472	.034	.477

Hardy-Weinberg Equilibrium

The frequency of genotypes at each polymorphic locus (0.95 criterion: a locus is considered polymorphic if the most common allele has a frequency of 0.95 or less) was tested for compliance with Hardy-Weinberg equilibrium expectations (see Appendix A). A chi-square test is suspect in cases where expected frequencies of some classes are low (see Sokal and Rohlf 1969). Consequently, when more than two alleles are observed at a locus, BIOSYS-1 pools genotypes into three classes and repeats the chi-square test. Three genotype classes result: 1) homozygotes for most common allele, 2) heterozygotes for most common allele and one of the other alleles, 3) all other genotypes. The resulting chi-square value is used with 1 degree of freedom.

Nearly all polymorphic loci were found to be in compliance with Hardy-Weinberg equilibrium expectations (Appendix A); those cases where there appeared to be a significant deviation from Hardy-Weinberg expectations were invariably due to single mosquitoes that were monomorphic for seemingly rare alleles. In such instances, it is likely that these individuals represent sampling error, mistaken readings of electromorphs on gels or possible null alleles.

F-Statistic Analysis

This analysis is a procedure for quantifying the genetic differentiation of populations by F-statistics (see Wright 1965, 1978; Nei 1977). Wright's F_{st} value, or fixation

index, provides a measure of the genetic variation in the population that is attributable to subpopulations; i.e., departure from panmictic expectations of allele frequencies within sub-populations relative to those of the entire population. When F_{st} values are found to be significant (by chi-square analysis), this indicates significant genetic separation among these subpopulations. The chi-square test for significance of gene frequency differences at each locus among subpopulations is:

$$X^2 = 2NF_{st}(K-1)$$

with (K-1) (s-1) degrees of freedom, where N is the total sample size, K is the number of alleles for the locus, and s is the number of populations (Workman and Niswander 1970).

In this study, differences in allele frequencies between sub-populations were evident among samples from different geographic areas (e.g. California versus Washington). It was not clear, however, whether frequency differences were significant among proximate subpopulations within geographic areas. The four most proximate populations within an apparently homogenous ecological zone (central Sacramento Valley) were Sacramento (site 6), Knights Landing (site 8), Millers Landing (site 9) and Williams (site 10). All sites were within a 80 km radius from each other and showed the most similar allele frequencies observed in this study. F_{st} values were calculated for three of the most polymorphic loci among these four populations: Got-1, Mpi-1 and Pgm-1 (Table 5-9). Both Pgm-1 and Mpi-1 had F_{st} values that were

Table 5-9. Summary of F-statistics for genotypic frequency distributions at the Got-1, Mpi-1, and Pgm-1 loci in: All populations sampled in Oregon and Washington (sites 22, 23, 24, 25); all populations sampled in California (sites 2, 3, 6, 8, 9, 10, 12, 13, 15, 18); four proximate populations within the Sacramento Valley (sites 6, 8, 9, 10); a population from the Sacramento Valley (site 6) and one from Utah (site 26); a population from Yakima, Washington (site 25) and from Utah (site 26).

		Oregon + Washington	California	Sac. Valley (sites 6, 8, 9, 10)	Sac., CA + Utah	Yakima, WA + Utah
	F_{st}		.049	.004	.014	
Got-1	Chi sq		481.31	13.63	23.52	
	p		.001	.700	.001	
	F_{st}	.033	.119	.014	.246	.030
Mpi-1	Chi sq	544.02	785.41	50.80	503.01	25.68
	p	.001	.001	.001	.001	.001
	F_{st}	.011	.026	.015	.125	.091
Pgm-1	Chi sq	48.56	271.91	56.07	100.22	31.21
	p	.001	.001	.001	.001	.001

highly significant ($P < 0.001$). When F_{st} indexes were calculated for all populations within California, all three loci had highly significant values. F_{st} values forsubpopulations in Washington and Oregon were also highly significant. Highly significant F_{st} values were also obtained when Utah was clustered with a population from either California or Washington.

Since a frequency cline in the A allele (Rf = 100) at the Pgm-1 and Mpi-1 (Rf = 100) loci was apparent in Oregon and Washington, F_{st} values were calculated among all pairs of samples (Table 5-10). F_{st} values for Mpi-1 were significant between all pairs. F_{st} values for the Pgm-1 locus were significant in all instances, except between Madras and Hermiston (both within Oregon).

Table 5-10. Summary of F-statistics at the Pgm-1 and Mpi-1 loci for pairs of samples collected in Madras and Hermiston, Oregon (sites 22, 23), and Richland and Yakima, Washington (sites 24, 25).

site	Pgm-1			Mpi-1		
pair	F_{st}	Chi-sq.	P	F_{st}	Chi-sq.	P
Mad:Her	.010	4.62	.200	.066	32.34	.001
Mad:Ric	.039	29 72	.001	.154	167 86	.001
Mad:Yak	.046	18.22	.001	.380	209.76	.001
Her:Ric	.010	9.72	.050	.056	87.36	.001
Her.Yak	.014	11.26	.010	.094	218.70	.001
Ric:Yak	.001	1.10	.700	.083	149.40	.001

Genetic Distance and Identity Values

One of the most widely used measures of genetic distance is the D value of Nei (1978). This value expresses the probability that a randomly chosen allele from each of two different populations will be identical, relative to the probability that two randomly chosen alleles from the same population will be identical. When two populations are identical, the the identity value I = 1 and the genetic distance D = 0. The BIOSYS-1 program calculates D and I values for all possible combinations of pairs of populations.

In this study, D and I values between all pairs of populations show that genetic distance between them is low (Table 5-11). Even the population at Jasper Ridge, which was identified as An. hermsi (see Chapters 3 and 4), had I and D values that were indistinguishable from the conspecific I and D values of An. freeborni. All four proximate populations within the Sacramento Valley (sites 6, 8, 9, 10) had values of D = 0. Overall, the samples from Yakima and Utah produced the greatest genetic distance values when paired with all other population samples.

A cluster analysis was generated using the unweighted pair group method with arithmetic averaging (described by Sneath and Sokal 1973). The phenogram shows three main clusters (Fig. 5-8). All the California populations, except for Camino, are clustered together. The Yakima, Washington, sample shows more genetic divergence from the other

populations in Washington and Oregon than it does to the sample from Utah.

Table 5-11. Genetic distance and identity values for populations of Anopheles freeborni. Below diagonal: Nei (1978) unbiased genetic distance: above diagonal: Nei (1978) unbiased genetic identity.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 NICHLAND WA	*****	977	988	000	083	985	991	988	064	050	089	000	089	975	994
2 HERMISTON, OR	003	*****	988	000	000	992	995	991	000	001	000	000	988	979	988
3 YAKIMA WA	000	012	*****	974	985	979	981	977	975	977	975	975	975	967	991
4 MADRAS, OR	012	008	020	*****	983	991	992	994	991	991	001	992	988	979	979
5 JASPER OR	010	014	053	015	*****	908	994	000	975	000	991	000	988	975	979
6 Sacramento, CA	012	009	025	008	004	*****	1 000	1 000	1 000	1 000	988	908	994	963	975
7 MILLERS L ,CA	008	007	010	008	005	000	*****	1 000	1 000	1 000	986	908	908	000	000
8 WILLIAMS CA	012	010	020	000	004	000	000	*****	988	? 908	988	908	975	000	975
9 KNIGHTS L ,CA	015	011	027	000	000	000	000	001	*****	1 908	1 000	000	975	000	978
10 TEHAMA CA	012	000	023	009	000	000	000	000	908	*****	000	000	000	991	991
11 CHICO, CA	015	012	029	000	000	000	001	000	000	001	*****	000	975	983	979
12 CLEAR LAKE, CA	015	010	025	000	000	001	001	001	000	001	000	*****	993	988	975
13 PLEASANT, CA	015	012	025	012	012	000	008	000	001	000	991	000	*****	991	977
14 CAMINO, CA	024	021	034	003	008	010	017	015	010	010	017	020	008	*****	009
15 UINTAH, UT	000	012	991	021	020	021	017	021	003	000	020	003	024	030	*****

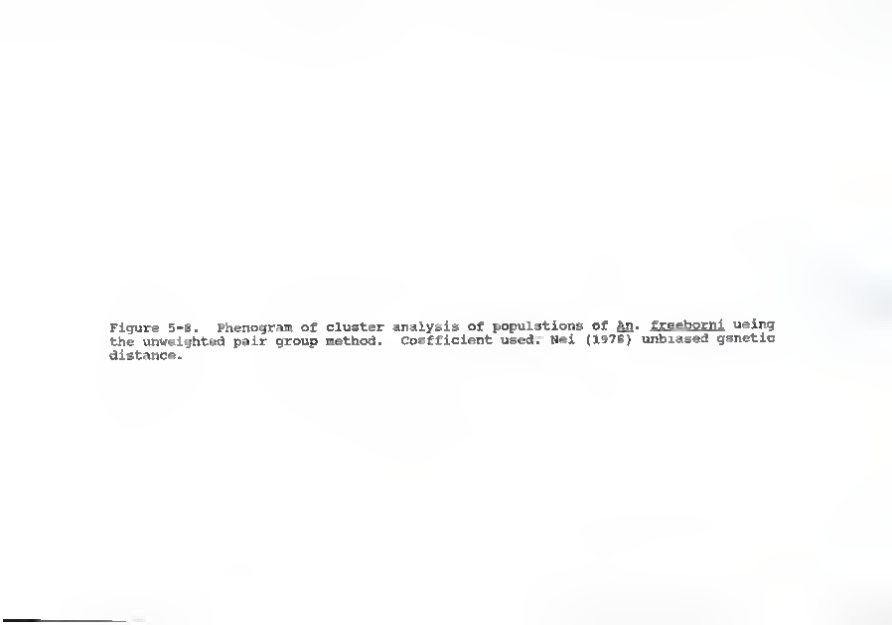
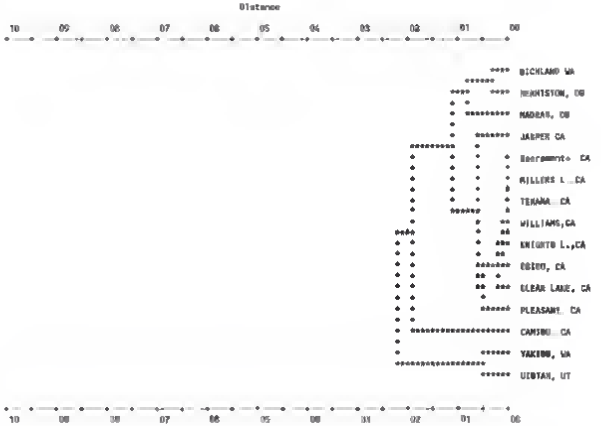


Figure 5-8. Phenogram of cluster analysis of populations of An. freeborni using the unweighted pair group method. Coefficient used: Nei (1978) unbiased genetic distance.


Distance
10 09 08 07 06 05 04 03 02 01 00
BICKLAND WA
MANTISTON, OR
MADRAS, OR
JASPER CA
Sacramento CA
MILLERS L..CA
TEHAMA CA
WILLIAMS,CA
KNIGHTS L.,CA
DIXON, CA
CLEAR LAKE, CA
PLEASANT CA
CAMBRU..CA
YAKIMA, WA
UINTAH, UT
10 09 08 07 06 05 04 03 02 01 00

Discussion

Anopheles freeborni appears to have relatively low levels of genetic variability, particularly for a species that is so widespread and inhabits such a variety of ecological zones. The majority of loci analyzed in this study are monomorphic, and even of those that are polymorphic only two or three (Mpi-1, Got-1 and Pgm-1) have three or more alleles. However, when we consider those loci that were polymorphic, but not used in the genetic analysis (because of unreliable scoring of electromorphs), the level of heterozygosity in this species approaches that found in other insects (Nevang 1980, Nevang et al. 1989).

Like Fujioka (1986), who did an enzyme electrophoresis comparison of An. hermsi and An. freeborni, this study did not find diagnostic loci that distinguish these two sibling species. Apparently, these two species can only be distinguished, at present, by hybridization or rDNA analysis (see Chapters 3 and 4). The genetic differentiation between both species is no greater than that found intraspecifically for An. freeborni.

There is obvious genetic substructuring of An. freeborni throughout its range. In general, the differentiation between populations reflects differences in ecological zones (e.g. Sierra Nevada vs Sacramento Valley) or geographic distance. In California, the Sacramento Valley and the Sierra Nevada foothills seem to harbor populations that are distinctive both by their X chromosome type and in allozyme

frequencies. However, even within each of these two zones, populations can have significantly different frequencies of certain alleles. Camino and Pleasant Valley Rd (sites 2, 3), for example, are only 13 km apart, but do not have identical allele frequency profiles (Table 5-6). Proximate populations collected from areas near Sacramento differed significantly in the F_{st} values obtained for the Mpi-1 and Pgm-1 loci.

In Chapter 3, significant differences in the frequency of the inversion on chromosome are 3R for proximate sites in the Sacramento Valley suggested that these populations are semi-isolated. The evidence from enzyme electrophoresis supports this hypothesis, since F_{st} values are highly significant at the Pgm-1 and Mpi-1 loci.

In Washington and Oregon, there is a cline in the frequency of the A (Rf = 100) and E alleles (Rf = 124) at the Mpi-1 locus. The A allele is very rare in Madras (site 22), but is the most common allele in Richland (site 24) and Yakima (site 25). The reverse is true for the E allele. The frequency cline of both alleles have a startling correlation to the frequency cline of the two X chromosome karyotypes in these populations (Table 5-12). Since many females from each site were identified chromosomally and then electrophoresed, it was possible to associate the type of X chromosome from an individual mosquito with its alleles at the Mpi-1 locus (Table 5-13).

It is obvious that there is a disproportionately higher frequency of the A allele associated with the standard X

Table 5-12. The frequency of the A allele (Rf = 100) at the Mpi-1 locus, and the frequency of the standard X chromosome for populations of Anopheles freeborni in Washington and Oregon.

		Frequency		
Population	n	A allele	n	Std X Chromosome
Madras	14	0.04	15	0.00
Hermiston	35	0.36	47	0.28
Richland	95	0.61	73	0.62
Yakima	55	0.90	50	0.90

chromosome; the reverse is true for the E allele. It is tempting to assert that the association of the A allele with the standard X karyotype demonstrates that the Mpi-1 locus is within the inversion region. Single cross-over events within inversions produce non-viable cross-over products, and thus the term "cross-over suppressors" has been used to describe the effects of inversions. The extent of cross-over suppression depends on the length of the inversion, its position in an arm, and on the sensitivity of the chromosome to rearrangement. But, an inversion can also effect the cross-over rate of loci outside of the inversion itself (Roberts 1976). This is particularly true for loci near the

inversion breakpoints, but is not limited to these areas. Consequently, although it is a reasonable guess that the Mpi-1 locus lies within the X inversion region (which

Table 5-13. The frequency of alleles at Mpi-1 for homokaryotypes of the standard X chromosome, homokaryotypes for In(X)A, and heterokaryotypes for populations of Anopheles freeborni sampled in Washington and Oregon. The allele frequencies determined from all individuals electrophoresed from each site is given in parenthesis.

Site = Madras

Allele Freq.	Frequency of X Chromosome Karyotype: Standard n=0	Heterokaryotype n=0	Inversion n=10
A (0.04)	--	--	0
C (0.00)	--	--	0.10
D (0.00)	--	--	0
E (0.46)	--	--	0.40
F (0.29)	--	--	0.20
G (0.21)	--	--	0.30

Site = Hermiston

Allele Freq.	Frequency of X Chromosome Karyotype: Standard n=3	Heterokaryotype n=11	Inversion n=18
A (0.36)	0.66	0.40	0.22
C (0.01)	0	0.05	0
D (0.01)	0	0	0
E (0.50)	0.17	0.45	0.64
F (0.10)	0.17	0.05	0.14
G (0.01	0	0.05	0

Table 5-13 continued

Site = Richland

Allele Freq.	Frequency of X Chromosome Karyotype: Standard n=21	Heterokaryotype n=27	Inversion n=4
A (0.81)	0.83	0.53	0
C (0.00)	0	0	0
D (0.01)	0	0	0
E (0.23)	0.15	0.30	0.50
F (0.15)	0.02	0.18	0.50
G (0.01)	0	0	0

Site = Yakima

Allele Freq.	Frequency of X Chromosome Karyotype: Standard n=21	Heterokaryotype n=4	Inversion n=0
A (0.90)	1.00	0.82	--
C (0.01)	0	0	--
D (0.00)	0	0	--
E (0.04)	0	0.13	--
F (0.04)	0	0.25	--
G (0.02)	0	0	--

comprises 2/3 of the total length of the chromosome), it is not certain.

A pericentric inversion reduces the rate of cross-over progeny, but generally does not prevent cross-over products altogether. Double cross-overs within the inversion, although rarer than single cross-over events, do produce viable gametes. Therefore, pericentric inversions merely reduce the rate of recombination of inversion protected

blocks of genes. Over time, the frequency of a particular allele within the inversion would be the same as that on the non-inverted region. This is similar in concept to linkage disequilibrium between tightly linked loci; over time, alleles at each locus should be randomly associated (unless other factors are contributing to apparent linkage disequilibrium e.g. migration, emigration, selection).

Several hypotheses can be invoked to explain why the A allele at the Mpi-1 locus is not equally represented in both X chromosome karyotypes. If we assume that the standard X karyotype is actually the derived inversion of a progenitor X chromosome then: 1) The A allele represents a recent mutation within the inversion and has increased in frequency (selective advantage?), 2) the allele mutated at the moment when the inversion was created and has since been represented in the alternative X karyotype by double cross-over events, 3) at the time the inversion was created, the MPI-1 locus coincidentally had the A allele, 4) two or more populations fixed for alternative arrangements of the X chromosome inversion and different alleles at the Mpi-1 locus have recently overlapped in their distribution. If we assume that the inversion X karyotype (e.g. that found in populations from Jeeper Ridge, Madras, Hermiston) was derived from the standard X karyotype (that type found throughout the Sacramento Valley), then the hypotheses listed above would apply to the Z allele.

If the A allele originated in or has a selective advantage in the standard X karyotype, then we might expect to see a pattern of association between this allele and the standard X chromosome karyotype throughout the distribution of An. freeborni. The A allele is the most common allele at the Mpi-1 locus in Yakima, Washington. It is also found in almost all populations within the Sacramento Valley and is very common in the sample collected in Utah. All of the above populations share the same X chromosome karyotype (standard). Those populations that have a high frequency, or are the fixed for the inversion karyotype have no A allele (Camino), or have a very low frequency of the A allele (Clear Lake). Anopheles hermsi at Jasper Ridge is also fixed for the X inversion and has no A allele at the Mpi-1 locus.

Although it is not possible to ascertain the origin of the A or E allele with a particular karyotype, it is plausible that the Washington and Oregon populations have recently overlapped in distribution (fourth hypothesis). Anopheles freeborni at Yakima, Washington may have been, at some point in the past, completely isolated from populations in Oregon that were fixed for the inversion on the X chromosome and had no A allele (but had a high frequency of the E allele). An example of such a population may be that found in Utah (site 26), which is fixed for the standard X karyotype, is almost fixed for the A allele and has no E allele. If populations of An. freeborni in Washington have

only recently overlapped with those in Oregon, then not enough time has elapsed for the A and E alleles at the Mpi-1 locus to be assorted with each X karyotype randomly. The relatively recent agricultural exploitation and development of these areas of Washington and Oregon could easily explain opportunities for sympatry of formerly isolated mosquito populations.

CHAPTER 6

GENERAL DISCUSSION

Anopheles freeborni is believed to be widely distributed throughout the states west of the Rocky Mountains (Carpenter and La Casse 1955). In this study An. freeborni was collected throughout the north and central parts of California, some areas of Oregon and Washington, and two locations in Utah. Within each state, An. freeborni may be found at a wide range of altitudes and a variety of ecological zones. In California, for example, these mosquitoes can be collected from marshes in Bishop [site 21, 1,270 m above sea level (m.a.s.l.)], ponds in the Sierra Nevada foothills (sites 2 and 3, 850 m a s.l.), areas around Clear Lake in the coastal range (site 15, 400 m.s.m.l.), and ricefields in the Sacramento Valley (sites 1-14, 10-150 m.a.s.l.).

There are various chromosome inversions and allozymes at some loci that have frequency differences among populations sampled in this study . For the most part, these differences can be related to geographic distance between two or more populations, or ecological zone differences. In California, for example, X chromosome karyotype frequencies seem to have a pattern of sorts. In valleys, whether at high or low altitude (e.g., Owens and

Sacramento Valleys), only the standard homokaryotype is present. Populations in hilly or mountainous zones are either polymorphic for the X inversion or may be fixed for the inversion homokaryotype (sites 2, 3, 15, 19; Camino, Pleasant Valley Rd., Clear Lake and Onyx). The presence of the inversion homokaryotype does not seem to be solely correlated with altitude, since mosquitoes collected in the Owens Valley (the highest sites in this study) had the standard X chromosome only. This digression from a purely altitudinal correlation was also observed in samples from Washington and Oregon.

Differences in chromosome inversion and allozyme frequencies are not unexpected between relatively distant or isolated populations of a species. Such populations would probably have minimal or no migration and, hence, no genetic exchange; selection and genetic drift could then generate interpopulation differences in allele and inversion frequencies.

If we accept the proposition that all populations sampled in this study represent the same species (except for the population at Jasper Ridge, which is An. hermsi), then it is reasonable to propose that the inversion homokaryotype has a selective advantage in hilly or mountainous zones. To speculate further on the possible sequence of events leading to present inversion patterns, the inversion on the X chromosome that occurred at some point in the past allowed

ons karyotype to invade either valley regions (standard X chromosome) or hilly areas (inversion X chromosome).

Did the standard karyotype give rise to the inversion karyotype, or vice versa? Although this study does not and cannot answer this question, there are some small 'hints' of information that may support the latter. In the first place, it would seem that An. hermsi was derived from populations of An. freeborni that inhabit hilly or mountainous zones. Both share the same X chromosome karyotype and have similar or overlapping ecological zones (e.g. both can be found in hilly areas or relatively close to the coast). In addition, An. hermsi, although homosequential to An. freeborni, does not appear to have any polymorphic inversions; it is a subset of those found in An. freeborni. It is generally conceded that the progenitor of a new race, type or species will often be more polymorphic.

Those populations of An. freeborni that have the inversion homokaryotype are generally less polymorphic than populations found within the Sacramento Valley. Here again, populations either fixed or primarily composed of inversion homokaryotypes seem to have a subset of the polymorphism found in populations fixed for the standard karyotype. It would seem, then, that populations of An. freeborni with the standard X karyotype were the progenitors of the inversion type. The inversion perhaps conferred a selective advantage that allowed the formation of sub-populations able to invade foothill and mountainous regions. As stated by Coluzzi et

al. (1979), an evolutionary consequence of optimal habitat choice in individuals carrying an inversion might be their differential probability of shifting to new or marginal ecological niches. "This would mean differential probabilities of forming isolates from which genetic reorganization can be initiated, thus providing a more coherent way of explaining the frequent involvement of inversions in speciation."

APPENDIX A

Chi square test for deviation from Hardy Weinberg equilibrium for polymorphic loci of populations of An. freeborni collected from various sites in Washington, Oregon, California and Utah. Levene (1949) correction for small sample size employed in chi square analyses. Pooled chi-square analysis are shown only for loci not otherwise in Hardy-Weinberg equilibrium

Population: BICHLAND, WA (site 24)

locus	class	observed number	expected number	chi square	DF	P
Acon-1						
	A A	81	80 251			
	A B	7	8 547			
	A C	1	.950			
	B B	1	.261			
	B C	0	.050			
	C C	0	.000			
				3 513	3	.319
Sod 1						
	B B	0	.000			
	B E	1	.990			
	B-E	0	.010			
	B B	99	99 .515			
	B E	2	1 .980			
	B-E	0	.005			
				.015	3	.999
Pk-2						
	A A	78	76 .905			
	A-C	15	17 .100			
	C-C	2	.005			
				1 .621	1	.001
Mal-1						
	A A	80	34 .683			
	B B	0	.608			
	B E	24	28 .164			
	B-F	15	17 .057			
	A G	0	1 .217			
	B B	0	.608			
	D D	0	.000			
	D B	0	.226			
	D-F	1	.148			
	D-S	0	.011			
	D-R	0	.005			
	E E	4	4 .778			
	E F	9	6 .370			
	E G	1	.455			
	E-B	1	.228			
	F F	1	2 .000			

Locus	Class	Observed number	Expected number	Chi square	DF	P
Mpi-1	F-G	1	296			
	F-H	0	148			
	G-I	0	.005			
	G-K	0	.011			
	H-K	0	.000			
				15.136	15	.442
Pgi-1						
	A A	93	92.268			
	A B	9	10.463			
	B B	1	.268			
				2.206	1	.137
Pgm-1						
	A-A	64	65.692			
	A B	35	31.659			
	A D	0	.791			
	A J	4	3.166			
	B-B	2	3.697			
	B D	1	.190			
	B L	0	.758			
	D-D	0	.000			
	D-L	0	.019			
	E-L	0	.028			
				8.456	6	.374

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D
Acon-1	0	9.547	-.157	-.162
Got-1	3	2.980	.012	.007
Hk-2	15	17.190	-.125	-.127
Mpi-1	52	53.534	-.024	-.029
Pgi-1	9	10.463	-.136	-.140
Pgm-1	40	36.583	.089	.095

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Population, WERNLSTOB, PA (size 25)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1						
	A A	20	20.154			
	A B	2	1.877			
	A C	2	1.877			
	A D	1	.936			
	B B	0	.015			

Locus	Class	Observed number	Expected number	Chi square	DF	P
	B C	0	.062			
	B-B	0	.031			
	C C	0	.015			
	C D	0	.031			
	D D	0	.009			
				.175	6	.999
Sat_1						
	A-A	0	.007			
	A B	2	1.972			
	A-E	0	.014			
	B B	68	68.021			
	B E	1	.986			
	E E	0	.000			
				.022	3	.999
Mpi_1						
	A-A	3	4.348			
	A C	0	-.362			
	A-D	1	.362			
	A E	14	12.681			
	A-F	3	2.536			
	A D	1	.362			
	C C	0	.009			
	C D	0	.014			
	C E	1	.897			
	C F	0	.101			
	C G	0	.014			
	D D	0	.009			
	D E	0	.507			
	D F	0	.101			
	D G	0	.014			
	E-E	0	0 633			
	E-F	4	3 551			
	E G	0	.507			
	F-F	0	.304			
	F G	0	.101			
	G G	0	.000			
				5 404	15	.987
Pgi_1						
	A A	47	47 097			
	A B	1	.961			
	A-D	4	3 845			
	B B	0	.000			
	B D	0	-.039			
	D D	0	-.058			
				.105	3	.991
Pep_1						
	A A	29	26 368			
	A B	16	20 811			
	A D	2	2 051			
	A 1	1	1 387			
	B B	6	3 919			
	B D	1	.811			
	B 1	1	.541			
	D D	0	.027			
	D 1	0	.054			
	1 1	0	-.009			
				3 118	6	.794

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D
Acon-1	5	4 815	+ 054	050
Got-3	3	2 972	-.017	010
Mpi-1	24	21 725	.121	.105
Pgi-1	9	6 845	.062	.032
Pgm-1	21	25 685	.175	.104

Population: YAKIMA, WA (site 25)

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Est-2						
	A-A	37	37.515			
	A-B	1	.921			
	A-C	1	.921			
	A-D	8	5 528			
	B-B	0	.000			
	B-C	0	.011			
	B-E	0	.067			
	C-C	0	.000			
	C-E	0	.067			
	D-D	0	.169			
				.571	A	.000
Mpi-1						
	A-A	47	44 505			
	A-C	1	.968			
	A-D	3	3 633			
	A-F	1	3 633			
	A-S	0	1 817			
	C-C	0	.000			
	C-E	0	.037			
	C-F	0	.037			
	C-G	0	.018			
	D-D	0	.255			
	D-F	1	.147			
	E-D	0	.073			
	F-F	1	.055			
	F-S	0	.073			
	S-D	1	.000			
				132 468	10	< 001
Pgm-3						
	A-A	28	24 444			
	A-D	0	7 111			
	D-D	4	.444			
				36 073	1	< 001
Pgi-1						
	A-A	68	66 235			
	A-B	3	2 843			
	A-D	4	3 791			
	A-F	2	1 895			
	B-B	0	020			
	B-D	0	073			
	B-F	0	039			
	D-D	0	039			

Locus	Class	Observed number	Expected number	Chi-square	DF	P
	B-F	0	.052			
	F-F	0	.007			
				1.362	6	.969
Dpn-1						
	A A	50	50.000			
	A B	22	21.774			
	A D	3	3.226			
	B B	2	2.265			
	B D	1	.497			
	D-D	0	.059			
				.220	3	.974

Chi-square test with pooling

Locus	Class	Observed number	Expected number	Chi square	DF	P
Mpi-1	Homozygotes for most common allele	87	44.525			
	Common/rare heterozygotes	5	9.991			
	Rare homozygotes and other heterozygotes	3	.505	16.974	1	< .001

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	P
Est-2	6	7.017	-.074	.064
Mpi-1	6	10.376	.416	.422
Pgm-2	0	7.111	1.000	1.000
Pgi-1	9	8.699	-.041	.038
Dpm-1	26	25.697	.010	.012

Population: MADRAS_OR (A15)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Aprn-1						
	A A	9	9.848			
	A B	6	4.727			
	A C	2	1.576			
	B B	0	.455			
	B-C	0	.364			

Locus	Class	Observed number	Expected number	Chi-square	DF	P
	C C	0	.030			
				1 378	3	.711
Got 1						
	A A	1	030			
	A B	0	1 939			
	B B	19	15 030			
				35 032	1	< 001
Mpi 1						
	A-A	0	000			
	A E	0	681			
	A F	1	896			
	A E	0	222			
	E-E	4	2 869			
	B-F	4	3 852			
	B C	1	2 858			
	F F	1	1 857			
	F E	1	1 770			
	B D	2	556			
				8 140	6	228
Pgm-1						
	A A	0	7 927			
	A B	0	10 146			
	E-E	4	2 927			
				993	1	319

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation Index (F)	D
Acon 1	0	6 667	-.236	200
Got 1	0	1 939	1 000	1-000
Mpi 1	7	9 519	-.237	365
Pgm 1	E	10 146	192	-.212

Population: JASPER CA (site 19)

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Est 2						
	A-A	33	33 133			
	A C	2	1 893			
	A-E	3	2 842			
	C C	0	015			
	C E	0	050			
	E E	0	068			
				169	3	986
Got 1						
	B B	32	31 047			
	B E	24	24 419			
	B H	1	696			
	B 1	1	1 595			

Locus	Class	Observed number	Expected number	Chi square	DF	P
	B-J	0	1.395			
	B-B	3	4.412			
	B-I	0	.271			
	B-I	1	.543			
	B-J	0	.543			
	B-B	0	.009			
	B-I	0	.016			
	B-J	0	.016			
	I-I	0	.009			
	I-J	0	.031			
	J-J	1	.009			
				129.984	10	< .001
Mdh-1						
	A-A	65	65.503			
	A-B	7	8.503			
	A-F	4	3.009			
	B-B	1	.232			
	B-F	0	.232			
	F-F	0	.039			
				3.543	3	.305
Mpi-1						
	C-C	0	.000			
	C-D	0	.008			
	C-B	1	.427			
	C-F	0	.476			
	C-B	0	.009			
	C-J	0	.010			
	D-D	1	.146			
	D-E	2	2.562			
	D-F	1	2.854			
	D-B	1	.175			
	D-J	0	.058			
	B-E	13	8.104			
	E-F	13	27.932			
	B-B	0	1.282			
	B-J	0	.427			
	F-F	15	11.617			
	F-B	2	1.427			
	F-J	1	.476			
	B-B	0	.029			
	B-J	0	.029			
	J-J	0	.000			
				18.602	15	.232
Pgp-3						
	A-A	32	32.043			
	A-C	3	2.913			
	C-C	0	.043			
				.046	1	.830
Pgp-5						
	A-A	27	23.430			
	A-F	5	7.274			
	A-I	0	4.049			
	F-F	2	.693			
	F-I	0	.740			
	I-I	3	.205			
				49.431	5	< .001

Chi-square test with pooling

Locus	Class	Observed number	Expected number	Chi square	DF	P
Got-1	Homozygotes for most common allele	32	31.947			
	Common/rare heterozygotes	26	27.907			
	Rare homozygotes and other heterozygotes	7	6.047	.310	1	.578
Pep-5	Homozygotes for most common allele	27	23.438			
	Common/rare heterozygotes	5	12.123			
	Rare homozygotes and other heterozygotes	5	1.438	13.546	1	<.001

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	Q
Est-2	5	4.813	-.053	.039
Got-1	27	29.326	.072	.578
Mdh-1	11	12.226	.094	.100
Mdh-2	23	31.223	.256	.263
Pep-2	3	2.913	.045	.030
Pep-5	5	12.863	.806	-.411

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Population- Sacramento, CA (site 6)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1						
	A A	51	50.308			
	A B	3	2.795			
	A C	2	3.726			
	A D	2	1.663			
	B B	0	.026			
	B C	0	-.103			
	B D	0	-.051			
	C C	1	.001			
	C D	0	.005			
	D D	0	.000			
				15.642	8	.005
Est-2						
	A A	35	34.259			
	A B	2	1.852			
	A C	2	3.704			

Locus	Class	Observed number	Expected number	Chi square	DF	P
	A F	1	.926			
	B B	0	.012			
	B C	0	.099			
	B F	0	.025			
	C C	1	.074			
	C F	0	.049			
	F F	0	.000			
				12 577	6	050
Mol 1						
	A-A	4	1 124			
	A B	8	10 491			
	A E	4	9 905			
	A-N	0	118			
	A-S	0	257			
	B-B	43	38 112			
	B E	10	22 260			
	B N	1	.675			
	B L	0	1 349			
	E E	4	3 124			
	E B	0	195			
	E-L	2	391			
	B B	0	000			
	B 1	0	012			
	1 1	0	000			
				19 650	10	033
Mol 1						
	A A	0	437			
	A-C	0	.477			
	A-G	2	1 067			
	A-E	8	3 515			
	A F	0	2 940			
	A D	1	356			
	A B	1	079			
	A J	0	018			
	A-N	0	238			
	C C	0	099			
	C D	2	954			
	C E	3	1 907			
	C-F	1	1 470			
	C B	0	278			
	C-B	0	040			
	C J	0	159			
	C B	0	119			
	D D	2	1 628			
	D E	11	7 629			
	D F	5	9 881			
	D B	0	1 113			
	D-N	0	139			
	D-J	0	634			
	D B	0	477			
	E E	5	7 470			
	E F	14	11 782			
	E B	3	2 225			
	E N	0	318			
	E J	2	1 272			
	E-N	0	954			
	F F	5	4 411			
	F B	2	1 715			
	F B	0	245			

Locus	Class	Observed number	Expected number	Chi square	DF	P
Mpi-1	F-J	1	.980			
	F-M	1	.735			
	G-G	0	.539			
	G-H	0	.046			
	G-J	1	.185			
	G-G	0	.139			
	H-H	0	.000			
	H-J	0	.026			
	H-P	0	.020			
	J-J	0	.040			
	J-M	0	.079			
	M-M	1	.020			
				75.593	36	< .001
Pgi-1	A-A	62	62.075			
	A-B	1	.970			
	A-D	6	5.900			
	B-B	0	.000			
	B-D	0	.030			
	D-D	0	.045			
				.026	3	.994
Pgm-1	A-A	55	55.179			
	A-B	5	5.255			
	A-D	11	10.511			
	A-H	1	.876			
	B-B	0	.108			
	B-D	1	.497			
	B-E	0	.041			
	D-D	0	.455			
	D-E	0	.085			
	E-E	0	.000			
				1.267	6	.975

Chi square test with pooling

Locus	Class	Observed number	Expected number	Chi square	DF	P
Got-1	Homozygotes for most common allele	43	38.112			
	Common/rare heterozygotes	28	37.775			
	Rare homozygotes and other heterozygotes	14	9.112	5.778	1	.016
Mpi-2	Homozygotes for most common allele	5	7.470			
	Common/rare heterozygotes	38	33.060			
	Rare homozygotes and other heterozygotes	33	35.470	1.727	1	.189

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D
Acon-1	7	8.607	.188	.187
Est-2	5	6.654	.259	.249
Got-1	34	42.633	.198	.202
Mpi-1	58	51.854	-.126	.119
Pgi-1	5	4.880	-.058	.025
Pgm-1	10	17.262	.050	.043

Population- MILLERS L ,CA (site 03)

Locus	Class	Observed number	Expected number	Chi square	df	P
Acon-1						
	A-A	48	48.193			
	A-B	3	2.885			
	A-C	2	1.820			
	A-D	1	.945			
	A-O	1	.945			
	B-B	0	.038			
	B-C	0	.055			
	B E	0	.028			
	B O	0	.028			
	C-C	0	.009			
	C-E	0	.018			
	C O	0	.010			
	D O	0	.000			
	O O	0	.000			
				216	10	.999
Est-2						
	A A	45	43.808			
	A B	6	5.394			
	A C	2	5.394			
	B B	0	.138			
	B C	0	.332			
	C C	2	.138			
				27.921	5	< .001
Got-1						
	A-A	0	.569			
	A B	11	8.394			
	A E	2	2.088			
	A 1	0	.095			
	A-K	0	.205			
	B B	38	35.409			
	B O	11	15.999			
	B 1	0	.725			
	B K	1	2.168			
	B B	4	1.880			
	E-1	1	.161			
	E K	0	.482			
	1 1	0	.008			

locus	class	observed number	expected number	chi square	DF	P
	I B	0	.022			
	B B	1	.022			
				56 033	10	< 001
Mpi-1						
	A A	1	.270			
	A C	0	.135			
	A D	0	.811			
	A E	2	1 216			
	A F	0	1 351			
	A B	1	.676			
	A J	0	.270			
	C C	0	.000			
	C D	1	.162			
	C E	0	.245			
	C F	0	.270			
	C B	0	.135			
	C J	0	.054			
	D D	1	.405			
	D E	2	1 459			
	D F	0	1 622			
	D B	1	.811			
	D J	0	.324			
	E E	1	.973			
	E F	2	2 432			
	E B	0	1 216			
	E J	1	.486			
	F F	2	1 216			
	F B	3	1 351			
	F J	1	.541			
	B B	0	.270			
	B J	0	.270			
	J J	0	.027			
				18 603	21	.611
Pgm-1						
	A A	62	63 129			
	A B	4	3 165			
	A D	13	13 453			
	A E	2	1 583			
	A L	7	5 548			
	B B	0	.043			
	B D	0	.489			
	B E	0	.050			
	B L	0	.201			
	D D	2	.278			
	D E	0	.245			
	D L	0	.098			
	E E	0	.007			
	E L	0	.101			
	L L	0	.151			
				3.278	10	.948

Chi square with pooling

locus	class	observed number	expected number	chi square	DF	P
Est-2	Homozygotes for most common allele	45	43 606			
	Common/rare heterozygotes	8	10 789			
	Rare homozygotes and other heterozygotes	2	.606	3.977	1	.046

Locus	Class	Observed number	Expected number	Chi square	DF	P
Got 1	Homozygotes for most common allele	38	35 609			
	Common/rare heterozygotes	23	26 182			
	Rare homozygotes and other heterozygotes	0	5 409	2.384	1	.123

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D
Acon 1	7	6 771	-.043	.034
Est 2	8	11 110	.274	.281
Got 1	26	31 314	.164	.178
Mai 1	14	15 899	.092	.118
Pgm-1	26	25 691	-.019	.012

****

Population WILLIAMS CA (alle 18)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1						
	A A	46	46.574			
	A B	3	2 713			
	A C	4	3.617			
	A E	3	2 715			
	A G	2	1 809			
	B B	0	.026			
	B C	0	.104			
	B E	0	078			
	B G	0	052			
	C C	0	052			
	C E	0	104			
	C G	0	078			
	E E	0	026			
	E G	0	052			
	G G	0	009			
				702	10	999
Est 2						
	A A	57	57 165			
	A B	7	6 669			
	B B	0	.165			
				192	1	661
Got 1						
	A A	1	675			
	A E	10	8 645			
	A E	1	3 023			

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Est_1	A A	0	.101			
	A I	0	.292			
	A B	28	27.823			
	A E	1A	19.535			
	A G	0	.651			
	A I	2	1.302			
	E E	0	3.372			
	E B	1	.233			
	E I	0	.668			
	B G	0	.000			
	B I	0	.016			
	I I	0	.000			
				0.962	10	536
Est_2	A A	0	.644			
	A C	1	.945			
	A D	2	1.981			
	A E	7	4.520			
	A F	4	4.151			
	A G	1	1.698			
	A J	0	.283			
	A B	0	.094			
	C C	0	.283			
	C-D	1	1.321			
	C-E	5	3.019			
	C F	2	2.767			
	C-G	1	1.132			
	C-J	0	.189			
	C-B	0	.063			
	D D	0	1.321			
	D-E	4	8.340			
	D-F	11	5.811			
	D G	1	2.377			
	D-J	2	.396			
	D B	0	.132			
	E E	7	7.894			
	E F	9	13.383			
	E G	7	5.434			
	E J	1	.808			
	E B	1	.302			
	F F	6	5.950			
	F G	6	4.981			
	F J	0	.830			
	F-B	0	.277			
	G G	1	.962			
	G-J	0	.340			
	G-B	0	.113			
	J J	0	.019			
	J B	0	.019			
	B B	0	.000	24.308	28	.664
Pgm_1	A A	50	50.916			
	A-D	7	7.644			
	A E	1	1.771			
	A G	0	.885			
	A I	3	2.656			
	A J	1	1.771			
	D D	0	.214			
	D E	0	.322			
	D G	0	.061			
	D I	0	.183			
	D J	1	.122			
	E E	0	.009			

Locus	Class	Observed number	Expected number	Chi square	DF	P
	E G	1	.015			
	E I	0	.046			
	E J	0	.031			
	G G	0	.000			
	G I	0	.023			
	G J	0	.015			
	I I	0	.023			
	I J	0	.046			
	J J	0	.000			
				72.229	15	<.001

Chi square test with pooling

Locus	Class	Observed number	Expected number	Chi square	DF	P
Pgm-1	Homozygotes for most common allele	92	90.916			
	Common/rare heterozygotes	12	14.158			
	Rare homozygotes and other heterozygotes	2	.916	1.638	1	.201

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	G
Acon-1	12	11.313	-.070	.061
Est-2	7	6.649	-.058	.058
Got-1	30	33.992	-.111	+-.117
Mpi-1	66	63.731	.042	.036
Pgm-1	14	14.832	.049	.056

Population 061GBT0 L - CA (site 8)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1						
	A A	43	42.617			
	A B	6	3.589			
	A C	3	2.692			
	A D	2	3.589			
	A E	1	.897			
	B B	0	.056			
	B C	0	.112			
	B D	0	.158			
	B E	0	.037			

Locus	Class	Observed number	Expected number	Chi square	DF	P
	C C	8	968			
	C-D	0	112			
	C F	0	026			
	D D	1	.856			
	D E	0	.037			
	D F	0	960			
				17.251	10	968
Est 2						
	A A	20	80.007			
	A B	3	2 867			
	B B	0	.067			
				973	1	.787
Got 1						
	A A	2	882			
	A B	10	9 706			
	A E	1	3 151			
	A K	0	378			
	B B	21	24 968			
	B E	22	18 174			
	B K	3	1.941			
	B F	1	2 521			
	E K	0	.630			
	K K	0	.025			
				8 042	6	235
Mpi 1						
	C C	0	120			
	C D	2	.960			
	C E	0	720			
	C F	1	.840			
	D D	0	.240			
	D E	1	1 920			
	D F	3	2 240			
	E E	0	.640			
	E F	1	.600			
	F F	2	1 960			
	E E	1	.480			
	F F	0	960			
	F E	1	960			
	E E	0	640			
				5 705	10	839
Pgi 1						
	A A	58	56 421			
	A B	2	1 854			
	A C	2	1 854			
	A D	7	8 489			
	B B	0	.067			
	B C	0	.029			
	B D	0	.102			
	C C	0	.007			
	C D	0	.102			
	D D	0	.955			
				447	8	998
Pgm 1						
	A A	42	41 394			
	A B	8	9 372			
	A D	13	12 496			
	A E	1	.731			
	B B	1	.482			
	B D	1	1 411			
	B E	0	068			

Locus	Class	Observed number	Expected number	Chi-square	DF	P
	D-D	1	.876			
	D B	0	.117			
	B B	0	.008			
				1.000	6	.986

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation Index (f)	D
Acon-1	10	11.243	.102	.111
Mdh-1	3	2.867	- .070	.047
Got-1	36	31.985	.135	.126
Mpi-1	11	10.280	- .113	.078
Pgd-1	11	10.431	.062	.055
Pgm-1	24	24.255	.003	.011

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Population: IENAMA, CA (site 13)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1						
	A A	42	42.617			
	A B	2	1.796			
	A C	10	8.972			
	B B	0	.000			
	B C	0	.107			
	C C	0	.421			
				.767	3	.867
Est 2						
	A A	17	17.144			
	A-B	4	3.737			
	B B	0	.966			
				.171	1	.679
Got-1						
	A A	3	1.736			
	A B	15	13.537			
	A C	0	3.972			
	B B	24	24.010			
	B C	13	14.826			
	C C	4	2.091			
				6.843	3	.077

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Sp1-1						
	A A	1	.480			
	A C	0	.480			
	A D	0	1.560			
	A E	4	2.640			
	A F	1	2.760			
	A G	0	.480			
	A H	0	.120			
	C C	0	.080			
	C D	0	.693			
	C E	3	1.173			
	C F	1	1.227			
	C G	0	.213			
	C H	0	.053			
	D D	1	1.840			
	D E	4	3.815			
	D F	4	5.907			
	D G	0	.693			
	D H	1	.173			
	E E	3	3.080			
	E F	2	4.747			
	E G	1	1.173			
	E H	0	.293			
	F F	5	3.373			
	F G	3	1.227			
	F H	0	.307			
	G G	0	.080			
	G H	0	.053			
	H H	0	.000			
				25.648	21	.221
Pgm-1						
	A A	47	46.224			
	A B	1	2.592			
	A D	11	11.232			
	A D	1	.864			
	A J	1	.864			
	B B	1	.624			
	B D	0	.312			
	B D	0	.624			
	B J	0	.024			
	D D	1	.624			
	D B	0	.104			
	D J	0	.104			
	D D	0	.000			
	D J	0	.000			
	J J	0	.000			
				41.532	10	< .001

Chi square test with pooling

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Pgm-1	Homozygotes for most common allele	47	46.224			
	Common/rare heterozygotes	14	15.552			
	Rare homozygotes and other heterozygotes	2	1.224	.840	1	.417

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation Index (F)	S
Aconr 1	12	10 953	-.166	.096
Est 2	4	3 707	-.104	.079
Est 3	30	32 355	.065	.073
Mpi 1	28	29 867	.050	.043
Pgm-1	14	16 128	.125	.132

Population: ENIGO CA (site 12)

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Aconr 1						
	A A	34	33 346			
	A B	9	9 481			
	A C	1	1 827			
	B B	0	185			
	B C	1	148			
	C C	0	012			
				5 525	5	.137
Est 2						
	A A	12	12 037			
	A B	1	865			
	A D	1	963			
	D D	0	0ee			
	B G	0	037			
	D G	0	096			
				.040	3	.096
Est 3						
	A A	2	1 766			
	A B	11	11 039			
	A E	2	2 429			
	B B	15	15 909			
	B F	8	7 143			
	F E	0	.714			
				1 356	3	716
Mpi 1						
	A A	0	023			
	A C	0	186			
	A D	0	418			
	A F	0	326			
	A F	2	465			
	A F	0	558			
	C C	0	148			
	C+D	3	859			
	C F	0	681			
	C F	0	930			
	C D	1	1 118			
	D+D	1	837			

Locus	Class	Observed number	Expected number	Chi-square	DF	P
	D E	0	1.465			
	D F	3	2.093			
	D D	1	2.512			
	E E	2	.488			
	E-F	3	1.628			
	E D	0	1.953			
	F F	1	1.047			
	F D	0	2.791			
	D D	5	1.535			
				35.101	13	.002
Pgm-1						
	A A	31	30.959			
	A B	4	3.210			
	A D	12	10.454			
	A E	0	.804			
	A H	0	.804			
	A I	0	.804			
	B B	0	.062			
	B D	0	.536			
	B E	0	.041			
	B H	0	.041			
	B I	0	.041			
	D D	0	.804			
	D E	0	.134			
	D H	0	.134			
	D I	1	.134			
	E-E	0	.000			
	E H	1	.010			
	E-I	0	.010			
	H H	0	.000			
	B 1	0	.010			
	I I	0	.000			
				105.252	15	< .004
Kdh-1						
	A A	0	9.048			
	A C	2	1.905			
	C C	0	.048			
				.053	1	.818

Chi square test with pooling

Locus	Class	Observed number	Expected number	Chi square	DF	P
Mpi-1	Homozygotes for most common allele	5	1.535			
	Common/rare heterozygotes	2	8.930			
	Rare homozygotes and other heterozygotes	15	11.535	14.242	1	< .001
Pgm-1	Homozygotes for most common allele	31	30.959			
	Common/rare heterozygotes	16	10.862			
	Rare homozygotes and other heterozygotes	2	1.950	.001	1	.971

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation Index (F)	D
Acon-1	7	7.457	.059	-.061
Est-2	2	1.963	-.057	.019
Got-1	22	20.610	-.081	.067
Mdh-1	13	17.930	.258	-.275
Pgm-1	18	17.175	-.059	.048
Xdh-1	2	1.905	.105	.051

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Population: CLEAR LAKE, CA (site 15)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1						
	A-A	46	45.882			
	A-B	7	6.176			
	A-C	4	5.294			
	A-E	2	1.765			
	B-B	0	.176			
	B-C	0	.353			
	B-E	0	.118			
	C-C	1	.126			
	C-E	0	.101			
	E-E	0	.008			
				7.274	6	.296
Est-2						
	A-A	30	30.495			
	A-B	14	11.558			
	A-C	1	2.473			
	B-B	0	1.000			
	B-C	0	.462			
	C-C	1	.033			
				31.238	3	< .001
Got-1						
	A-A	1	1.099			
	A-B	16	15.603			
	A-E	2	2.969			
	A-I	1	.530			
	B-B	52	51.675			
	B-E	21	19.932			
	B-I	0	2.215			
	E-E	2	1.838			
	E-I	0	.424			
	I-I	1	.816			
				66.097	6	< .001
Mdh-1						
	A-A	54	54.174			
	A-B	3	2.651			
	A-C	1	.950			
	A-D	2	1.001			
	A-E	1	.950			
	B-B	0	.025			

Locus	Class	Observed number	Expected number	Chi-square	DF	P
	B-C	0	.009			
	M B	0	.050			
	M M	0	.025			
	B-C	0	.000			
	C-C	0	.017			
	C B	0	.008			
	B B	0	.005			
	B M	0	.017			
	M M	0	.000			
				1.192	10	.000
Sp1 1						
	M M	0	.000			
	M C	0	.057			
	M B	1	.067			
	A E	0	.356			
	A F	0	.407			
	M B	0	.074			
	M M	0	.015			
	M J	0	.044			
	C-C	0	.074			
	C-B	1	.333			
	B-B	2	1.778			
	C F	2	2.037			
	C M	0	.370			
	C M	0	.074			
	C J	0	.222			
	B-B	1	.267			
	B E	3	3.200			
	B F	2	3.467			
	B B	0	.667			
	B M	1	.133			
	B J	0	.400			
	E E	9	8.356			
	E F	15	16.356			
	E-E	3	3.556			
	E M	0	.711			
	E J	3	2.133			
	F F	13	11.000			
	F B	3	4.074			
	F M	0	.815			
	F J	1	2.444			
	M B	1	.333			
	B-M	0	.148			
	B J	0	.444			
	M M	0	.007			
	M J	0	.096			
	J J	0	.111			
				32.942	28	.256
Pgi 1						
	A M	70	89.232			
	M B	2	1.897			
	M B	3	4.742			
	A E	2	1.897			
	B M	0	.006			
	B-B	0	.005			
	B E	0	.026			
	B B	1	.065			
	B E	0	.065			
	E E	0	.000			
				14.392	8	.000
Pgm 1						
	A A	61	60.000			
	A B	4	3.556			

Locus	Class	Observed number	Expected number	Chi square	DF	P
	A-C	0	.009			
	B-B	9	10.867			
	A-E	1	.009			
	B-B	0	.039			
	B-C	0	.020			
	B-D	0	.314			
	B-B	0	.020			
	C-C	0	.000			
	C-D	1	.078			
	C-E	0	.007			
	D-D	1	.431			
	D-E	0	.078			
	E-E	0	.000			
				15.384	10	.207

Chi square test with pooling

Locus	Class	Observed number	Expected number	Chi square	DF	P
Sdh-2	Homozygotes for most common allele	30	30.495			
	Common/rare heterozygotes	15	14.011			
	Rare homozygotes and other heterozygotes	1	1.495	.241	1	.623
Got 1	Homozygotes for most common allele	35	51.875			
	Common/rare heterozygotes	37	37.649			
	Rare homozygotes and other heterozygotes	7	6.875	.039	1	.865
Pgi 1	Homozygotes for most common allele	70	69.232			
	Common/rare heterozygotes	7	8.535			
	Rare homozygotes and other heterozygotes	1	.232	2.823	1	.093

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	P
Acon 1	13	13.807	-.051	.058
Est 2	15	14.473	.048	.036
Got 1	49	41.372	.028	.033

Coefficients for heterozygote deficiency or excess

Rad_1	7	6 793	.000	.000
Mpi_1	42	47 852	.916	-.122
Pgi_1	7	8 000	.189	-.195
Pgm_1	15	18 529	.087	-.093

Population: PLEASANT_CA (site 3)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon_1						
	A-A	12	12.103			
	A-B	2	1.662			
	A-E	1	.931			
	B-B	0	.098			
	B-E	0	.000			
	E-E	0	.000			
				.120	3	.989
Gpi_1						
	A-A	2	1.000			
	A-B	5	6.000			
	A-E	1	.400			
	B-B	5	4.200			
	B-E	0	.600			
	E-E	0	.000			
				1.841	3	.606
Mpi_1						
	A-A	11	16.128			
	A-B	1	2.744			
	B-B	1	.128			
				7.652	1	.006
Mpi_1						
	A-A	1	.067			
	A-C	0	.000			
	A-E	0	.467			
	A-F	0	.267			
	A-E	0	.133			
	E-E	2	1.000			
	E-E	2	2.000			
	C-F	0	.000			
	E-E	0	.000			
	E-E	1	.067			
	E-F	1	.667			
	E-D	0	.333			
	F-F	0	.067			
	F-D	1	.133			
	E-E	0	.000			
				29.380	10	.000

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Mpi-1						
	A-A	16	16.000			
	A-E	1	1.000			
	E-E	0	.000			
				.000	1	.999
Pgi-1						
	A A	17	17.027			
	A B	1	.973			
	A D	1	.973			
	B B	0	.000			
	B D	0	.027			
	D D	0	.000			
				.029	3	.999
Pgm-1						
	A A	12	12.103			
	A B	3	2.793			
	B B	0	.103			
				.120	1	.729

Chi-square test with pooling

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Mpi-1	Homozygotes for most common allele	2	1.000			
	Common/rare heterozygotes	2	4.000			
	Rare homozygotes and other heterozygotes	4	3.000	2.333	1	.127

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D
Acon-1	3	2.862	-.064	.948
Got-1	6	7.000	.189	-.143
Hex-1	1	2.760	.623	-.638
Mdh-1	4	6.200	.312	-.355
Pgi-1	2	1.975	-.041	.014
Pgm-1	3	2.793	.111	.074

Population CAMINO, CA (site 2)

Locus	Class	Observed number	Expected number	Chi-square	DF	P
Acon-1						
	A A	36	33.878			
	A-B	1	2.883			
	A-C	1	.961			
	B A	1	.039			
	B-C	0	.039			
	C-C	0	.000			
				25.000	3	< .001
Got 1						
	A A	1	.098			
	A B	2	4.746			
	A E	0	.063			
	B B	28	27.150			
	B E	1	.937			
	E E	0	.006			
				9.503	3	.023
Mpi 1						
	C C	17	16.682			
	C-B	6	5.765			
	C F	0	.824			
	C J	2	1.647			
	B-B	0	.412			
	B F	1	.157			
	B J	0	.275			
	F F	0	.000			
	F J	0	.039			
	J J	0	.020			
				7.078	6	.314
Pgi 1						
	A A	34	34.190			
	A B	5	4.684			
	A D	1	.937			
	B A	0	.127			
	B B	0	.063			
	B D	0	.000			
				2.17	3	.975
Pgm 1						
	A A	32	28.123			
	A B	3	5.037			
	A G	0	5.877			
	A K	1	.843			
	B B	0	.135			
	B G	A	.510			
	B K	0	.076			
	G G	A	.259			
	G K	0	.006			
	K K	0	.006			
				41.175	8	< .001

Chi square test with pooling

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1	Homozygotes for most common allele	36	35 078			
	Common/rare heterozygotes	2	3 844			

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1	Rare homozygotes and other heterozygotes	1	078	11 820	1	001
Gal-1	Homozygotes for most common allele	28	27 159			
	Common/rare heterozygotes	3	4 668			
	Rare homozygotes and other heterozygotes	1	159	5 089	1	024
Pgm-1	Homozygotes for most common allele	32	28 125			
	Common/rare heterozygotes	4	11 753			
	Rare homozygotes and other heterozygotes	5	1 123	99 625	1	< 001

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D
Acon-1	2	3 883	478	683
Gal-1	3	4 746	358	-368
Mpi-1	9	8 666	056	036
Pgi-1	6	5 604	069	056
Pgm-1	7	12 452	438	-437

Population: UINTAH, UT (site 26)

Locus	Class	Observed number	Expected number	Chi square	DF	P
Acon-1						
	A A	25	18 528			
	A C	4	4 943			
	C C	1	.528	561	1	.454
Est-2						
	A A	31	31 067			
	A E	1	.057			
	A B	3	2 870			
	E E	0	.000			
	E B	0	.043			
	B B	0	.043			
				.005	3	.992
Got-1	A A	1	.261			
	A B	2	4 000			
	A D	0	.030			
	A E	1	.848			
	B B	54	52 000			
	B D	1	.000			
	B E	21	22 000			
	D D	0	.000			
	D E	0	.170			
	E E	3	2 291			
				10 193	6	.013
Mpi-1						
	A A	34	34 060			
	A F	17	15 630			
	F F	1	1 620			
				.386	1	.534
Pgm-1						
	A A	26	27 485			
	A B	39	36 034			
	B B	10	11 069			
				.518	1	.472

Chi-square test with pooling

Locus	Class	Observed frequency	Expected frequency	Chi square	DF	P
Got-1	Homozygotes for most common allele	54	52 485			
	Common/rare Heterozygotes	24	27 200			
	Rare homozygotes and other Heterozygotes	5	3 800	1 178	1	.278

Coefficients for heterozygote deficiency or excess

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	p
Acon_1	6	6.943	-.120	.136
Est-2	6	5.870	.049	.034
Got-1	25	28.248	-.119	.115
Mpi-1	17	15.880	.095	.084
Pgm-1	39	36.034	.090	.082

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BIOGRAPHICAL SKETCH

Gary Nathan Fritz was born on "dia de las inocentes", December 28, 1953 in Waupun, Wisconsin. After tasting his first earthworm at the age of two, Gary decided that a life with invertebrates would be a noble and rewarding endeavour. Gary's parents attempted to wean him off 'bugs' by living everywhere except the tropics. His childhood was spent in La Paz, Bolivia, where his parents were Methodist missionaries. Junior and high-school years were spent in Champaign, Illinois and Santiago, Chile, respectively. In 1974, Gary chose to major in biology at Beloit College, Wisconsin. In 1978, Gary began his M.Sc. in the Entomology-Nematology Department at the University of Florida. Infused with his parent's nomadic instincts, Gary spent the next 7 years in Mexico, Costa Rica, Peru and England. In 1984, he returned to the University of Florida to pursue a Ph.D. in entomology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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